

Characterisation of the responses, and possible mechanisms behind, spontaneous phasic activity in the isolated guinea pig bladder.

Steven M Finney

**M.D. Thesis
The University of Edinburgh
Year of Presentation 2008**



UNIVERSITY OF EDINBURGH

ABSTRACT OF THESIS

Name of candidate: Steven Mark Finney

Address:

Degree: Medical Doctorate

Date: 28th June 2006

Title of thesis: Characterisation of the responses, and possible mechanisms behind, spontaneous phasic activity in the isolated guinea pig bladder.

Spontaneous and volume induced phasic activity in the isolated guinea pig bladder is generated and modulated by a complex interaction of opposing excitatory and inhibitory stimuli. Although the exact nature and location of these mechanisms are not known, they represent local mechanisms inherent to the wall of the bladder. It has been suggested that phasic activity represents a component of afferent activity involved in the generation of sensation. Evaluation of the effects of the M₃ specific muscarinic antagonist 4-DAMP have identified cholinergic mechanisms involved in the generation of pacemaker activity that are sensitive to the effects of M₃ antagonism. It is therefore suggested that antimuscarinics exert their clinical effects upon sensation through this mechanism.

The intrinsic characteristics of spontaneous, phasic activity in response to volume have also revealed a period of inhibition following volume reduction termed the inhibitory phase. This is dependant upon the magnitude and duration of volume increase prior to decrease and is regulated by a distinct mechanism comprising of a combination of excitatory and inhibitory stimuli co-ordinated by ganglia. It can be manipulated by anticholinergic medication, nicotinic antagonists and purinergic agonists suggesting its underlying generation and regulation to be complex.

The actions of hexamethonium and pancuronium upon phasic activity have suggested the presence of non-ganglionic nicotinic receptors involved in the modulation of spontaneous and volume induced activity; in addition to the role of ganglia in co-ordinating the return of activity during the inhibitory phase.

Purinoceptors were found to display a varied and complex response, both on pacemaker activity and upon the inhibitory phase. Both excitatory and inhibitory receptors were present; however, individual subtypes seemed to display a differing degree of functional relevance between low and high volume. Though P2X and P2Y may have excitatory and inhibitory effects respectively, the overall effect of purinergic receptor stimulation upon phasic activity is inhibitory.

The data obtained during the course of these experiments illustrate the mechanisms involved in the generation and co-ordination of spontaneous activity to be complex. It highlights novel mechanisms through which acetylcholine and adenosine triphosphate may be exerting an effect, and may account for the therapeutic actions of anticholinergic medications. It also highlights potential mechanisms which may act as further therapeutic targets in the development of newer drugs for the treatment of OAB.

Dedication

I dedicate this thesis to my wife, Michelle, whose support during the course of this research was invaluable. I also dedicate this to my baby daughters, Abigail and Sophie, whose valiant attempts at keeping me awake most nights aided its speedy completion.

Acknowledgements

I am deeply indebted to Professor James Gillespie and Mr Laurence Stewart for all their help, support and advice during the course of this research.

Declaration

I, Steven Mark Finney, hereby declare that the work embodied in this thesis is the result of my own independent investigation. This is in accordance with the rule 3.1.14 of Edinburgh University Postgraduate Study Programme.

signed:.....

✓ Steven Mark Finney.

TABLE OF CONTENTS

Abstract.....	ii
Dedication.....	iii
Acknowledgements.....	iv
Declaration.....	v
Table of contents.....	vi
Abbreviations.....	ix

INTRODUCTION

1.1	Overview.....	1
1.2	Basic overview of bladder anatomy: the classical perspective	3
1.2.1	'Classical' views on bladder function.....	6
1.3	'Newer' views on bladder function.....	7
1.3.1	Components of afferent 'noise'.....	7
1.3.2	Potential cholinergic components to afferent noise.....	8
1.4	Cholinergic modulated non-micturition activity: a cholinergic motor/sensory system.....	9
1.4.1	Association between non-micturition activity and afferent discharge.....	10
1.4.2	Non-micturition activity in animal models of bladder dysfunction.....	11
1.4.3	Mechanisms generating non-micturition activity.....	11
1.5	Interstitial cells and intramural ganglia: potential structures involved in the regulation of 'Autonomous activity'.....	14
1.5.1	Interstitial cells.....	14
1.5.2	Ganglia.....	21
1.6	Autonomous activity: integrated physiology of the isolated guinea pig bladder.....	23
1.6.1	Effects of increasing intravesical volume.....	24
1.6.2	Effects of nitric oxide.....	24
1.6.3	Effects of noradrenaline.....	25
1.6.4	Effects of adenosine triphosphate.....	26
1.6.5	Effects of substance P and calcitonin gene related peptide.....	26
1.6.6	Intrinsic changes in spontaneous activity during changes in intravesical volume: the volume response.....	27
1.6.7	Overview of the human clinical relevance of observations of autonomous activity in animal models.....	32
1.7	The Overactive Bladder syndrome (OAB): a clinical perspective and role of antimuscarinic medications.....	35
1.7.1	Basic problem.....	35
1.7.2	Further complications.....	36
1.7.3	Effects of antimuscarinics upon muscle strips.....	38
1.7.4	Role of muscarinic receptors.....	38
1.7.5	In vivo animal models of bladder dysfunction.....	39
1.7.6	In vivo evidence from human studies.....	40
1.8	Summary.....	49
1.9	Experimental hypothesis.....	51

MATERIALS AND METHODS

2.1	Overview.....	54
2.2	Materials.....	55
2.2.1	Organ bath and associated equipment.....	55
2.2.2	Pressure recording and data acquisition system.....	56
2.2.3	Consumables.....	58
2.2.4	Muscarinic agonists.....	59
2.2.5	Muscarinic antagonists.....	59
2.2.6	Nicotinic antagonists.....	59
2.2.7	Purinoceptor agonist.....	60
2.2.8	Purinoceptor antagonists.....	60
2.3	Methods.....	61
2.3.1	Preparation of the isolated guinea pig bladder.....	61
2.3.2	Animal sacrifice.....	61
2.3.3	Dissection of the animal bladder.....	61
2.3.4	Cannulation of the animal bladder.....	62
2.3.5	Weighing of the animal bladder.....	62
2.3.6	Transferring of the bladder to the organ bath.....	63
2.4	Preparation of pressure recording equipment.....	64
2.4.1	Setup of recording equipment.....	64
2.4.2	Equilibration.....	64
2.4.3	Calibration of the transducer and data acquisition system.....	64
2.4.4	Recording of intravesical pressure.....	65
2.4.5	Assessment of minimal quality of experimental preparation.....	65
2.5	Manipulation of the guinea pig bladder.....	66
2.5.1	Augmentation of autonomous activity.....	66
2.5.2	Addition of drugs.....	66
2.5.3	Removal of drugs.....	66
2.5.4	Alteration of intravesical volume.....	67
2.5.5	Marking of time points.....	67
2.6	Data analysis.....	69
2.6.1	Number of data samples (n).....	69
2.6.2	Mean average, standard deviation (SD) and standard error of the mean (SEM).....	69
2.6.3	Paired Student's t-test.....	69
2.6.4	t-test assuming unequal variances.....	69
2.6.5	One way Analysis of variance (ANOVA).....	70
2.6.6	Two way Analysis of variance (ANOVA).....	70

RESULTS

3.1	Introduction.....	71
3.2	Interpretation of results.....	72
3.2.1	Definition and calculation of parameters common to spontaneous activity under iso-volumetric conditions and volume induced responses.....	72
3.2.2	Definition and calculation of parameters specific to spontaneous activity under iso-volumetric conditions.....	74
3.3	Effects of arecaidine and carbachol upon spontaneous activity.....	78
3.4	Effects of 4-DAMP and AF-DX upon spontaneous activity.....	87
3.5	Intrinsic characteristics of volume induced changes in spontaneous phasic activity: the volume response.....	99

3.5.1	Overview.....	99
3.5.2	Definition and calculation of parameters specific to the ‘volume response’.....	102
3.5.3	Differing effects of volume increase and decrease.....	104
3.5.4	Effects of duration of volume increase.....	110
3.5.5	Effects of agonist concentration.....	113
3.6	Effects of tolterodine upon the volume response.....	118
3.7	Effects of the nicotinic receptor antagonists, hexamethonium and pancuronium, on the volume response.....	123
3.7.1	Effects of hexamethonium on the volume response.....	123
3.7.2	Effects of pancuronium bromide on the volume response.....	128
3.7.3	Effects of hexamethonium and pancuronium on underlying augmented autonomous activity.....	133
3.8	Effects of α/β methylene ATP on the volume response.....	136
3.9	Effects of the purinergic receptor antagonists NF 279, MRS 2179 and PPADS on the volume response.....	142
DISCUSSION		
4.1	Introduction.....	147
4.2	The effects of the muscarinic agonists, arecaidine and carbachol, and the muscarinic antagonists, 4-DAMP and AF-DX, upon agonist induced phasic activity under iso-volumetric conditions.....	151
4.3	Intrinsic characteristics of the volume response.....	161
4.4	Effects of alterations in intravesical volume, duration of volume increase and agonist concentration.....	163
4.5	Effects of tolterodine upon volume induced changes.....	168
4.6	Effects of the nicotinic antagonists hexamethonium and pancuronium on volume induced activity.....	170
4.7	Effects of α/β methylene ATP on volume induced activity.....	173
4.8	Effects of the purinergic receptor antagonists NF 279, MRS 2179 and PPADS upon volume induced activity.....	176
4.9	Summary.....	180
FUTURE STUDY		
5.1	Future study.....	186
REFERENCES		
6.1	References.....	188
PUBLISHED PAPERS		
7.1	Papers published as a result of this study.....	211

Abbreviations

OAB	Overactive Bladder Syndrome
LUTS	Lower urinary tract symptoms
DO	Detrusor overactivity
M _x	Muscarinic receptor subtype <i>x</i>
CNS	Central nervous system
ATP	Adenosine triphosphate
NO	Nitric oxide
SU-IC	Suburothelial interstitial cell
MC-IC	Muscle coat interstitial cell
SM-IC	Surface muscle interstitial cell
IM-IC	Intra-muscular interstitial cell
Sub P	Substance P
CGRP	Calcitonin gene related peptide
SNP	Sodium nitroprusside
α/β MATP	α/β methylene ATP
ODQ	oxadiazole quinoxalin-1-one
4-DAMP	4-diphenylacetoxy-N-methylpiperidine methobromide
FDV	First desire to void
SDV	Strong desire to void
C _c max	Maximum cystometric capacity
VFOC	Volume of first overactive contraction
PFOC	Pressure of first overactive contraction
pdetmax(filling)	Maximum detrusor pressure during filling phase

pdetmax-oc	Maximum pressure of largest overactive contraction
Qmax	Maximum flow during micturition
pdetmax	Maximum detrusor pressure during micturition
pdetQmax	Detrusor pressure at maximum flow
pdet-uo	Detrusor pressure at urethral opening
pdet-uc	Detrusor pressure at urethral closure
Oxy.	Oxybutynin
Tolt.	Tolterodine
Trosp.	Trospium Chloride
Darif.	Darifenacin
inc.	Significant increase
dec.	Significant decrease
no change	No significant change
f_{ss}	Frequency at steady state
IF_{max}	Maximum initial frequency
P_{max}	Maximal underlying pressure
P_{ss}	Underlying pressure at steady state
A_{init}	Amplitude during initial phase
A_{ss}	Amplitude at steady state
F_{pre}	Frequency prior to volume increase
IF_{init}	Frequency immediately after volume increase
A_{pre}	Amplitude pre-volume increase
P_{shift}	Underlying pressure change
t_{inc}	Duration of volume increase

INTRODUCTION

Chapter One

1.1 Overview

The overactive bladder syndrome (OAB) is a symptom complex characterised by urgency, with or without urge incontinence, usually accompanied by frequency and nocturia (Abrams et al, 2002). It is a common condition, affecting nearly 100 million people in the western world (Milsom et al, 2001; Stewart et al, 2001) and although not life threatening it seriously affects quality of life and ability to work. These lower urinary tract symptoms (LUTS) become more prevalent in the aging population, where 40% over the age of 70 years are affected. Therefore, LUTS represents a major problem to individuals, health professions and society alike. More importantly however, as the population within the Western world is generally ageing, those affected by LUTS is set to increase significantly.

The total economic cost of this collection of OAB symptoms is high. In 2002 the costs in the USA were approximately \$12.7 billion (Hu et al, 2004). These have been estimated to rise to \$17 billion and €22 billion/ year by the year 2005. Approximately 25% of expenditure is spent on treatment (drug therapy, clinical consultation and surgery). Of those who suffer only 28% seek help, with only half of these currently receiving treatments. Less than 3% regain long lasting normal control. Therefore, the above costs may represent an under-estimation of a much larger problem.

The primary clinical problem behind OAB is that of an increased urge to pass urine, termed 'urgency'. Remarkably, despite the prevalence and costs involved, the mechanisms underlying increased urge are not fully understood. In order to simply describe the condition the term OAB has been introduced (Abrams et al, 2002); a

diagnosis based upon clinical symptoms. In many cases where patients with OAB are studied using conventional urodynamics sensations of urgency are correlated with rises in intra-vesical pressure, a condition classified as detrusor over activity (DO). In the remainder of patients, sensations of urgency are not accompanied by pressure changes. This situation is currently described as OAB in the absence of DO but previously has been known as 'sensory urgency'. The differences seen in the relationship between sensation and overactive detrusor activity may be indicative of different clinical states. Alternatively, it may represent the fact that the nature of the clinical condition is not fully appreciated.

Since the DO component of OAB is associated with contractions of the bladder smooth muscle (detrusor muscle), it was argued that drugs affecting contractility would alleviate symptoms. Activity in the detrusor muscle is initiated at muscarinic receptors (M_3) and drugs designed to target these M_3 receptors have been proven to be effective in decreasing urgency and incontinence, (Chapple et al, 2005). However, it has been suggested that, at therapeutic doses, these drugs do not significantly affect bladder contraction (Andersson and Arner, 2004; Andersson and Yoshida, 2003; Finney et al, 2006). Therefore, if this is so our understanding of the mechanisms through which antimuscarinics act, and in turn our understanding of the generation of the sensation of urge and urgency, is incomplete.

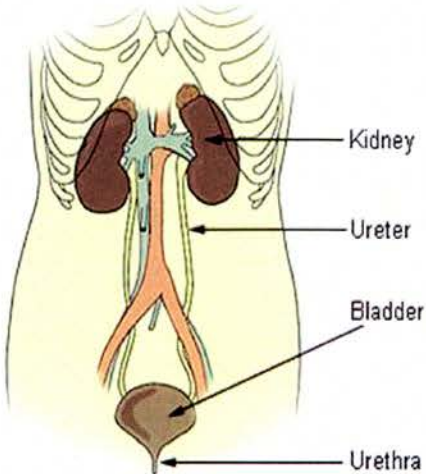
Thus, OAB is a major problem affecting a large number of people. The underlying causes are not fully understood and the economic costs are high. Pharmacological treatment, in the form of antimuscarinic medication, is available but the precise mode of action of these drugs is uncertain. It is therefore proposed that from these basic

clinical observations alone, alternative physiological processes need to be considered through which antimuscarinics may act, either wholly or in part. Furthermore, it also needs to be considered that additional mechanisms involved in the generation of sensation exist making afferent signalling more complex than traditionally thought.

1.2 Basic overview of bladder anatomy: the classical perspective

The bladder is a hollow, muscular organ situated in the pelvis in most mammalian species. It is distensible and acts as a reservoir to collect urine draining from both kidneys via the ureters, storing the urine till a time convenient for its expulsion. During urine expulsion, termed micturition, urine is expelled via the urethra. A diagram illustrating the relationship of the bladder within the human is illustrated, Figure 1a. The internal surface of the bladder is lined by transitional epithelium and is referred to as the urothelium. The urothelium is usually in the region of six cells thick and rests on a basement membrane. Immediately below the urothelium lies the lamina propria which is comprised of a thick layer of fibroelastic connective tissue that allows the considerable distension seen during bladder filling. Deeper still lies the smooth muscle of the bladder wall, termed the detrusor muscle layer. Three layers of detrusor muscle tend to exist; an inner longitudinal, middle circular and outer longitudinal layers. In the upper most parts of the bladder these three layers are not separable with fibres from each layer interlacing with those of different layers forming a meshwork of detrusor muscle. This meshwork of detrusor fibres is ideally suited for emptying a spherical bladder. A macroscopic example of these layers is illustrated, Figure 1b. A microscopic example of these layers is also shown, figure 1.

a) Components of the urinary system



b) Urinary bladder

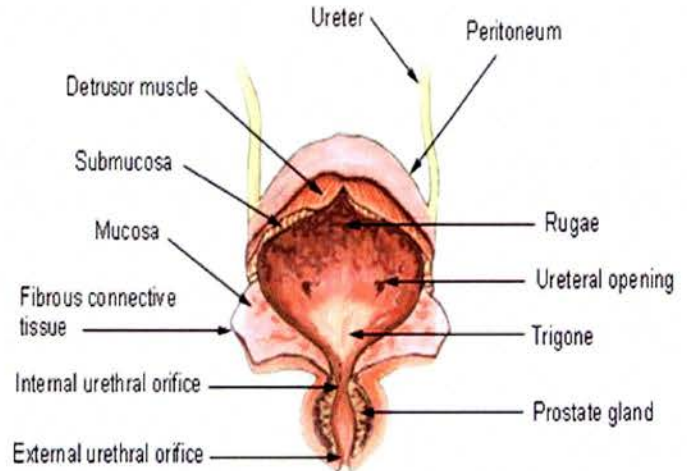


Figure 1: illustration highlighting the anatomy relating to the urinary bladder in the human. Figure 1a, illustrates the position of the bladder within the lower abdomen, connected to the kidneys on either side via the ureters. Urine drains from the kidneys down the ureters to the bladder where it is stored. Upon bladder contraction the urine is expelled, termed micturition, via the urethra. Figure 1b, illustrates the macroscopic relationship between the three layers of the bladder; urothelium (mucosa), lamina propria (submucosa) and the detrusor. The relationship of the ureters, prostate and urethra is also shown. (Illustrations have been reproduced subject to the regulations of the U.S. National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) Program).

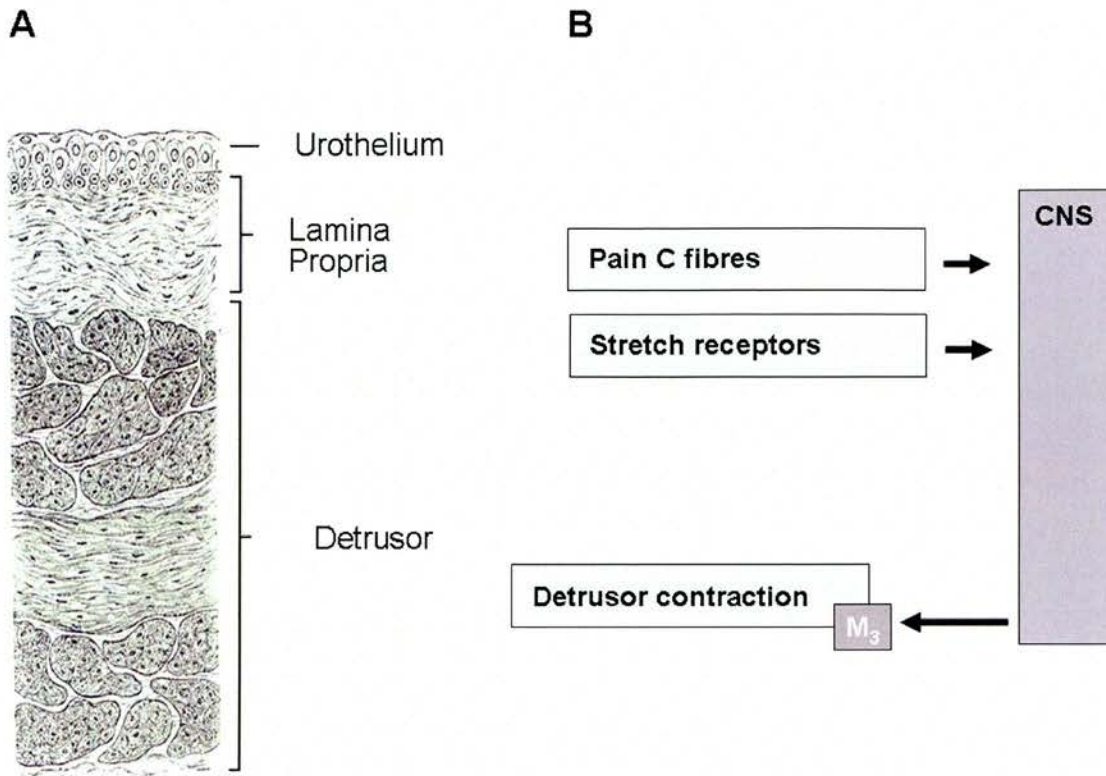


Figure 2: highlighting the classical structure and concept of bladder function during the filling phase. A, is a typical full thickness section of bladder tissue, (reproduction of a lithograph plate taken from Gray's Anatomy 20th edition, 1918). It can be seen that discrete layers exist comprising of urothelium, lamina propria and detrusor muscle. However, the limitations of reproductions obtained from light microscopy preclude the identification of the ultra structural architecture of the bladder. B, represents a schematic diagram of the classically described afferent and efferent input to the CNS.

Innervation of the bladder arises from autonomic fibres arising from the anterior portion of the pelvic plexus (alternatively known as the vesical plexus). These pass along the lateral and posterior ligaments to innervate the bladder. The bladder has a dense parasympathetic innervation with an abundance of postganglionic cell bodies, and is related to the mediation of detrusor contraction. Sympathetic innervation has also been identified though to a much lesser degree. However, the role of the sympathetic supply in the mediation of detrusor relaxation is uncertain.

1.2.1 'Classical' views on bladder function.

For many years views on the structure and function of the bladder were relatively simple. These entailed the bladder relaxing passively during filling, storing urine until a conscious decision is made for it to be expelled via the act of micturition. As urine accumulates, stretch receptors in the bladder wall send information on intravesical volume to the CNS. At an appropriate time, the CNS coordinates urethral sphincter relaxation and detrusor contraction resulting in voiding: termed the micturition reflex. Contractions arise from activation of postganglionic parasympathetic nerves mediated via the sacral parasympathetic nucleus resulting in the release of acetylcholine and excitation of smooth muscle via M₃ muscarinic receptors. This simple afferent/ efferent innervation is illustrated, figure 2b. In keeping with this view of function the 'classical' view of the structure of the bladder wall is also simple. As previously discussed, it comprises of the urothelium, lamina propria and muscle, with the urothelium acting solely as a protective barrier between the urine and the bladder wall.

However, as research into the bladder has evolved this description of physiological events and structure is no longer adequate. It is becoming apparent that inconsistencies

arise when attempting to explain the origins of urge, increased frequency or incontinence. Furthermore, no physiological roles are assigned to specific micro-anatomical structures in the bladder wall; such as the complex neural circuitry of the intra-mural ganglia, the diversity of neurotransmitter substances in the intramural nerves (sensory and motor), interstitial cells and the heterogeneous structure of the urothelium, lamina propria and muscle layers. Additionally, no roles are evident for the actions of urothelial signalling or non-micturition activity.

1.3 'Newer' views on bladder function

1.3.1 Components of afferent 'noise'

Over the past 60 years, data has been presented suggesting the presence of several sensory modalities originating from the bladder. In 1955, Iggo demonstrated firing in small myelinated A δ fibres in response to stretching of the bladder wall. In addition, small unmyelinated afferent fibres, C fibres, have also been identified that respond to excessive stretch, cold and noxious stimuli; probably contributing to painful sensations (Iggo, 1955; Morrison, 1998). More recently a complex system has been described with the potential to allow interaction between the urothelium and afferent C fibres. Adenosine triphosphate (ATP) and nitric oxide (NO) have been found to be released in response to mechanical distortion of the urothelium (Ferguson, 1999; Birder et al 1998, 2001, 2002). In addition, ATP has been shown to directly modulate firing of afferent bladder nerves (Rong et al, 2000). It has therefore been suggested that this urothelial system is involved in stretch induced modulation of bladder sensations (de Groat, 2004). However, the role of this system in the integrated physiology of the bladder is not known. But together, these observations suggest that afferent discharge arising from the bladder has different components: stretch mediated signals (mechanical), signals

emanating from noxious stimuli (pain) and signals modulated by chemicals released from the urothelium (chemical). Thus, it would appear that the sensory output from the bladder, 'afferent noise', consists of mechanical, pain and chemical components. A more complex schematic taking account of these newer modalities is illustrated, Figure 3.

These observations on specific structures/ response, however, have difficulty explaining cholinergic influence upon afferent activity. There is no doubt that the commercially available antimuscarinic drugs used in current clinical practice are able to reduce sensations of urgency by acting in the periphery and not in the CNS (Andersson and Arner, 2004). However, in the description of the components of 'afferent noise' described above, there are no elements that have been shown to involve acetylcholine or muscarinic receptors. Suggesting either that there are still unknown elements of these systems or that there are additional components of 'afferent noise'.

1.3.2 Potential cholinergic components to afferent noise

Data has been published however, suggesting additional responses involving cholinergic mechanisms to be present within the bladder linked to sensation. One possibility is that of muscarinic receptors on the urothelium (de Groat, 2004). Exogenous carbachol has been shown to increase micturition frequency and it was argued that muscarinic stimulation of the urothelium could lead to the release of ATP, the activation of afferent nerves and so modulate sensation (de Groat, 2004). Strong experimental evidence for this is not yet available and it is not clear what system would activate these receptors *in vivo*. However, Yoshida et al (2004) have demonstrated that

the urothelium is able to synthesise and release acetylcholine in response to stretch. It has been suggested that acetylcholine released in this manner could act on muscarinic receptors on sub-urothelial afferent nerves and so enhance firing. If this mechanism were present, it would be another example of 'chemical noise' and a potential site where antimuscarinic drugs could exert a therapeutic action. In relation to this idea it has been reported that instillation of the muscarinic agonist carbachol into the bladder increases micturition frequency (Kim et al, 2005). This could be brought about if carbachol activated muscarinic receptors on urothelium (de Groat, 2004 as above). Alternatively, carbachol could cross the urothelium and activate afferent nerves directly. From the structural perspective muscarinic receptors have been reported on nerve endings in the bladder (Somogyi et al, 1999). Where present, M_1 receptors are thought to be facilitatory while M_2 and M_4 inhibitory. A role for M_3 receptors has not been demonstrated. This conflicts with our current understanding, both clinically and pharmacologically, as the therapeutic actions of antimuscarinic drugs are thought to be M_3 specific. Though this would strongly suggest other cholinergic mechanisms to be present linked to sensation.

1.4 Cholinergic modulated non-micturition activity: a cholinergic motor/sensory system

Classical thinking dictates that the bladder is a low pressure compliant reservoir that is completely inactive during filling. However, the earliest cystometrogram on healthy young female volunteers, dating from 1882 (Mosso and Pellacani, 1882), clearly describe regular phasic rises in intravesical pressure during filling unrelated to the act of micturition. These observations were confirmed in the 1930s (Denny-Brown and Robertson, 1933) and 1960s (Plum, 1960) but their existence appears to have been

subsequently overlooked. Similar findings which have subsequently been termed ‘non-micturition activity’ have also been recorded in animal models. In the cat, the first observations were made by Sherrington in 1892 and confirmed on many occasions (Iggo, 1955; Gjone, 1965; Klevmark, 1977; Vaughan and Satchell, 1997).

1.4.1 Association between non-micturition activity and afferent discharge

The physiological role of this non-micturition activity is not known. However, it has been shown that, in the cat, these phasic rises in intravesical pressure are associated with bursts of afferent impulses thought to be emanating from rapidly adapting stretch receptors (Andersson and Yoshida, 2003; Vaughan and Satchell, 1995). Thus, this form of phasic motor activity may be part of a motor driven sensory system (Gillespie, 2004). If this is correct a further component to afferent noise can be introduced, that of ‘motor’ noise.

The amplitude and frequency of non-micturition activity alters during the filling phase (Klevmark, 1980; Vaughan and Satchell, 1995). Thus, if phasic activity is related to sensation then such changes suggest that the sensory outflow from the bladder could vary during the filling phase. It has been found that non-micturition activity can be modulated by both sympathetic and parasympathetic activity (Gjone, 1965; Vaughan and Satchell, 1992 and 1995). This is an important observation. If non-micturition activity can be modulated by efferent input to the bladder the implication is therefore that there is efferent control of afferent output: a modulated motor/sensory system (Gillespie, 2004). If modulation of such activity were to be altered the resulting effect would be to change sensations from the bladder: an increase in non-micturition activity

could thus represent one component to increased sensations of urgency (Gillespie, 2004).

1.4.2 Non-micturition activity in animal models of bladder dysfunction

A further indication to the potential importance of non-micturition activity may be implied from models of bladder dysfunction. Close examination of the published cystometry records of animals with experimentally induced increased micturition frequency, show an increase in non-micturition activity in the filling phase of the micturition cycle (Kim et al, 2005; Mitsui et al, 2001; Takeda et al, 2002). As postulated above, an increase in non-micturition activity would increase sensation and so trigger a micturition event earlier in the cycle; leading to increased frequency. It is difficult to be certain about the reliability of such records, since all of the experiments were undertaken using a single measurement of intra-vesical pressure (no independent measurements were made of intra-abdominal pressure to obtain true detrusor pressure). However, exaggerated and clear phasic rises in intravesical pressure can be seen in almost all of these models. Although not concrete by any means, such circumstantial evidence does however support the above arguments.

1.4.3 Mechanisms generating non-micturition activity

The mechanisms generating phasic activity *in vivo* are poorly understood. However, *in vitro* experiments, on the whole isolated bladder, allow some insight into the mechanisms in the bladder wall contributing to phasic activity. In 1892 Sherrington reported that phasic activity similar to non-micturition activity could be recorded in the isolated whole bladder. This observation has been confirmed in the isolated whole

guinea pig and rat bladder (Drake et al, 2003a; Drake et al, 2003b). The activity is intrinsic to the bladder wall and has been described as 'autonomous' activity. In the guinea pig, autonomous activity is associated with propagating waves of contraction spreading over the bladder surface accompanied by local areas of stretch. Autonomous activity can be augmented by nerve stimulation or direct application of muscarinic agonists: increasing concentrations of muscarinic agonist increase the amplitude and frequency of these small pressure increases termed transient contractions (Gillespie et al, 2004a). The initiation, coordination and control of this activity appear to be different from that involved with the micturition contraction. This has led to the proposition that there are two distinct systems capable of activating contractions in the detrusor: one associated with the global micturition contraction and the other the generation and regulation of phasic activity (Drake et al, 2003a).

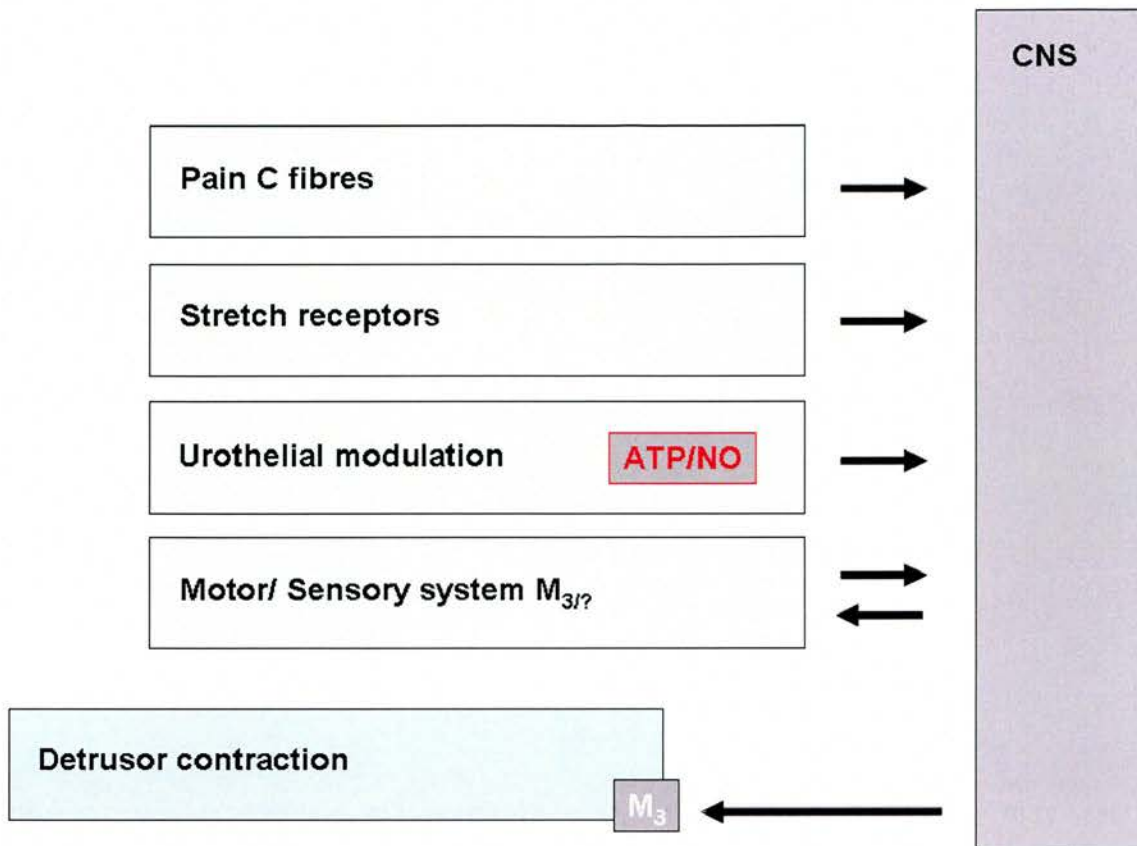


Figure 3: highlighting the potential components to afferent sensation arising from the urinary bladder. a) C fibre activity has been identified that responds to excessive stretch, cold and noxious stimuli. b) Stretch has been shown to lead to firing of $A\delta$ fibres. c) ATP and NO have been found to be released in response to mechanical distortion of the urothelium, with ATP release leading to direct firing of afferent nerves. d) A motor/ sensory system has been shown to exist whereby regular transient rises in intravesical pressure, unrelated to the act of micturition, are associated with transient increases in afferent activity. This activity increases in frequency with volume and can in turn be modulated via efferent parasympathetic/ sympathetic activity.

1.5 Interstitial cells and intramural ganglia: potential structures involved in the regulation of 'Autonomous activity'

It is unlikely that the local propagating waves of contraction are due to the direct spread of excitation from smooth muscle cell to smooth muscle cell. The trabecular organization of the muscle bundles would not allow a direct coupling between adjacent areas of muscle. Therefore, it has been postulated that there are specific structures involving specialized cells to distribute activity over the bladder surface. Two possibilities have been proposed. Firstly, a network of specialized cells, interstitial cells. Secondly, that intra-mural ganglia and nerves form a neural network to initiate and coordinate phasic activity (Drake et al, 2003a).

1.5.1 Interstitial cells

There are now a number of studies demonstrating the presence of a network of interstitial cells on the surface and within the outer muscle bundles of the detrusor (M^cCloskey and Gurney, 2002; Hashitani et al, 2004; Davidson and M^cCloskey, 2005; Gillespie et al, 2004), figure 4. These cells have been identified as cells which respond to nitric oxide with a rise in cGMP and by the expression of c-Kit, a specific marker of interstitial cells in the gut. However, at present there is no direct experimental evidence to demonstrate the exact role of interstitial cells within the bladder. Although, in the gut where the majority of work has been carried out into the roles of interstitial cells, they are thought to be responsible for the generation, co-ordination and propagation of peristalsis. Denervated intestinal loops have been found to exhibit spontaneous, rhythmical contractions (Huizinga JD et al, 1995), with increasing evidence to support that the Interstitial Cells of Cajal (ICC) are the underlying cells responsible for this

activity (Huizinga JD et al, 1995; Kobayashi S et al, 1995; Kobayashi S et al, 1996; Huizinga JD et al 1997; Sanders KM et al, 1999). Therefore, it is conceivable, given the similarities in spontaneous activity within the two organ systems that an analogous situation exists within the bladder.

Furthermore, the ureter also displays evidence of spontaneous, co-ordinated, rhythmical activity. These peristaltic waves are also analogous to peristalsis seen in the gut and originate within the renal pelvis, travelling distally towards the bladder, (Hannappel and Lutzeyer, 1978). Pacemaker cells, again similar to those characterised in the gut, seem to be implicated in this activity, (Klemm et al, 1999). Pharmacologically, this activity can be inhibited by the calcium channel blockers Nifedipine, Verapamil, and Diltiazem (Hertle and Nawrath, 1984). Indomethacin, a non-steroidal anti-inflammatory drug, has also been shown to reduce the frequency and amplitude of activity with PGE₂, a prostaglandin, markedly increasing it, (Thulesius and Angelo-Khattar, 1985).

The works of Lennon et al, have used animal models to illustrate the potential pathophysiological effects of bacterial infection, ureteric obstruction and ureteric stent insertion upon spontaneous ureteric activity. The bacteria *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* added to the lumen of the ureter was found to inhibit activity, whereas when added to the solution bathing the extra-luminal surface activity was increased, (Lennon et al, 1993a). Suggesting that systemic bacterial infection increases ureteric motility, whilst intraluminal urinary tract infection decreases it.

Further work has also shown that obstruction of the ureter inhibits activity, but this returns once the obstruction has been relieved, (Lennon et al, 1993b). Similarly, insertion of ureteric stents *in vivo* has been shown to reduce ureteric motility leading to the theoretical risk of delayed transit time for ureteric calculi, (Lennon et al, 1994).

It is now emerging that the network of interstitial cells seen within the bladder may be made up of a heterogeneous population. Based on location, interstitial cells are found in the sub-urothelial space (sub-urothelial interstitial cells: SU-ICs) and associated with the outer muscle layers of the bladder wall. In the guinea pig three types have been described: cells in the outer muscle coat (muscle coat interstitial cells: MC-ICs), cells on the surface of the muscle bundles (surface muscle interstitial cells: SM-ICs) and cells within the muscle bundles (intra-muscle interstitial cells: IM-ICs), (Gillespie et al, 2004). A further sub-population of cells may be identified from their expression of choline acetyltransferase (Gillespie et al, 2005). Examples of the three types of interstitial cells within the guinea pig bladder are illustrated, figures 5 to 7.

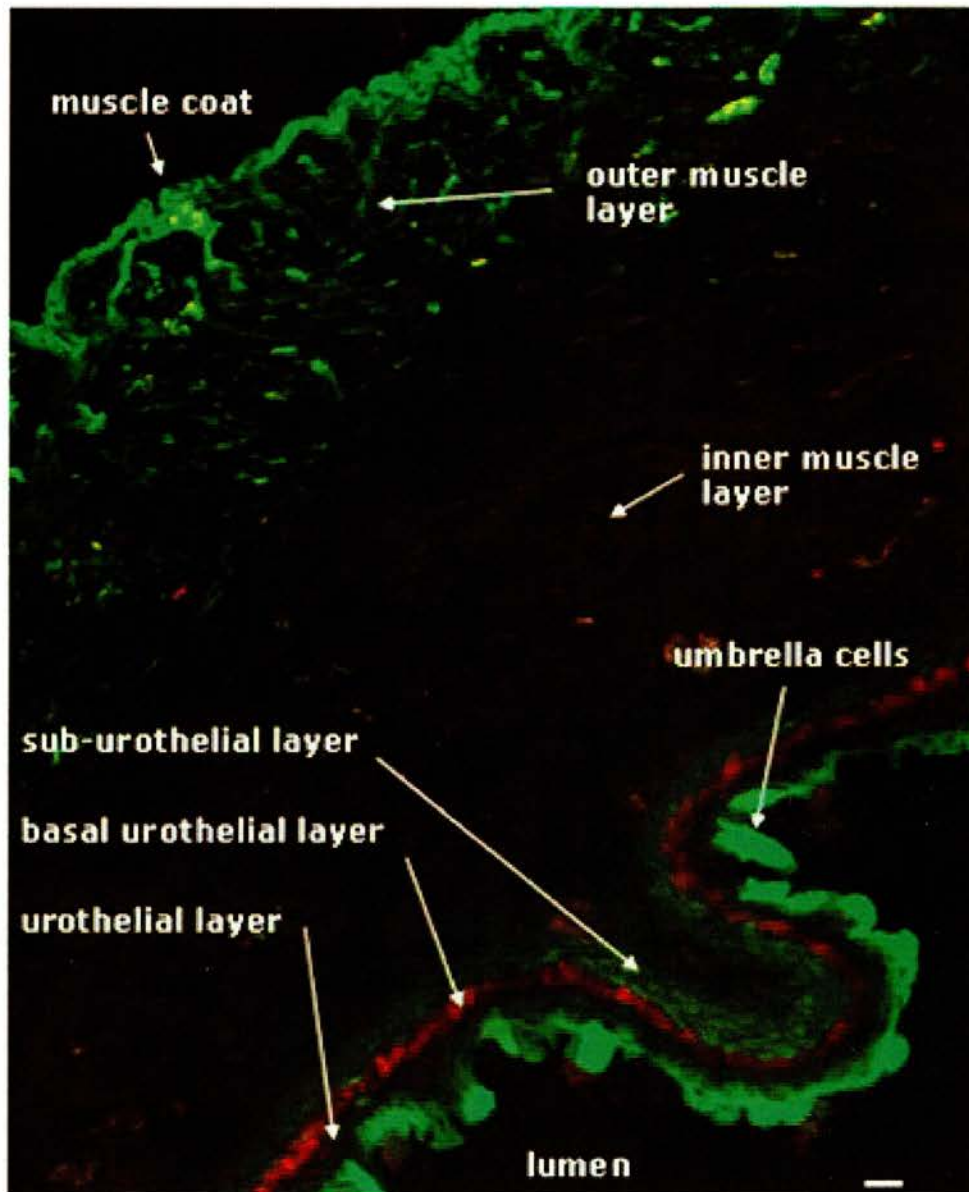


Figure 4: fluorescent image of a full-thickness section of the lateral wall of the bladder. cGMP staining illustrated as green fluorescence and nNOS (neuronal nitric oxide synthase) as red. cGMP positive cells are seen predominantly in the muscle bundles in the outer region of the bladder wall. Intense cGMP reactivity is seen in the umbrella cells of the urothelium and in a layer of cells immediately beneath the urothelium. A distinct layer of nNOS positive cells are visible in the basal layers of the urothelium. The horizontal bar at the bottom right corner = 10 μ m. Prior to staining the tissue was incubated in the non-specific phosphodiesterase inhibitor isobutyl-methyl-xanthene, followed by a further incubation in the nitric oxide donor NO₂ate. The above picture illustrates the presence of a suburothelial layer of interstitial cells, in addition to a dense network of interstitial cells in the outer muscle layers. (This illustration has been reproduced with permission from Professor James Gillespie and BJU International; Gillespie et al, 2005).

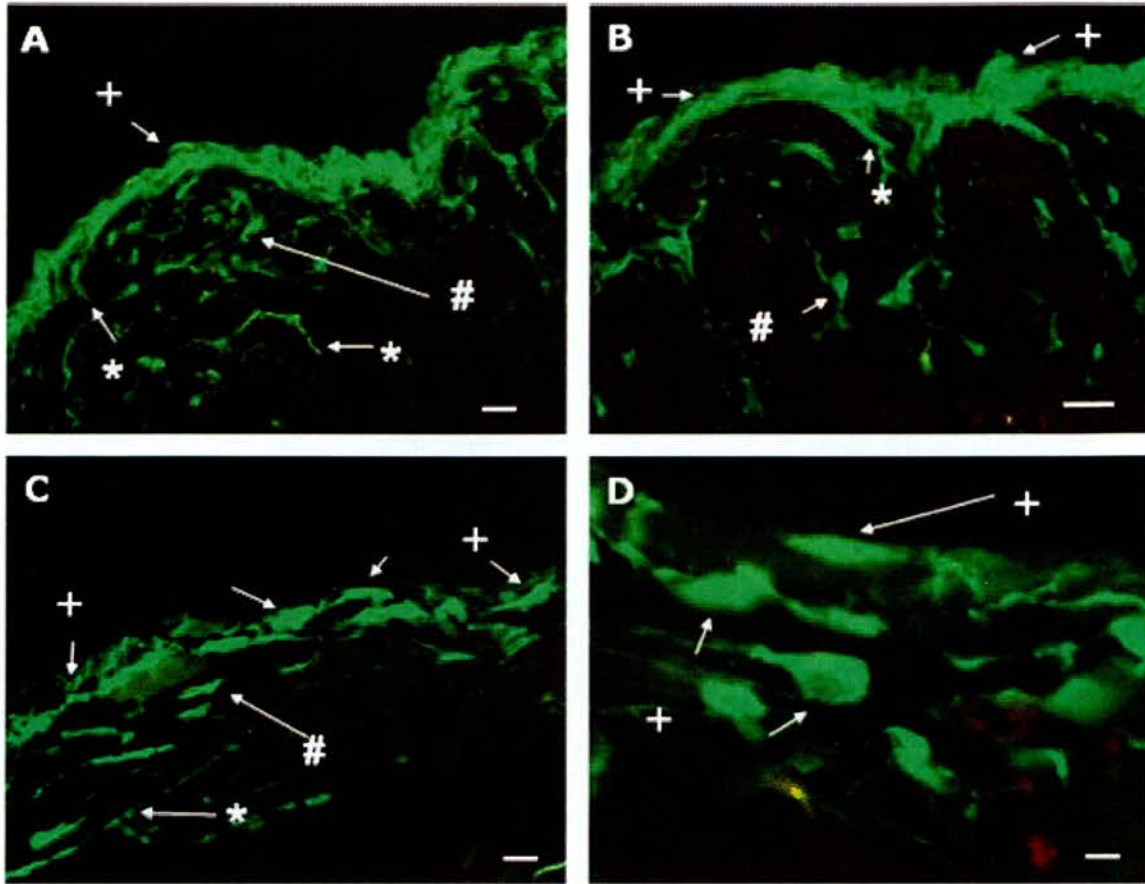


Figure 5: cGMP fluorescence is seen in the outer muscle coat. A and B illustrate dense staining on the outer muscle coat (+). Processes originating in the outer coat can be seen descending into to the underlying muscle bundles (*). Cells within the muscle bundles can also be seen (#). C and D show enlarged images of the muscle coat. These two panels show that the interstitial cells comprise of individual cells and connecting fibres (MC-IC). Horizontal bars in the bottom right represent 40µm in A and B, 15µm in C, and 8µm in D. Prior to staining the tissue was incubated in isobutyl-methyl-xanthene, followed by NO_nate (This illustration has been reproduced with permission from Professor James Gillespie and BJU International; Gillespie et al, 2005).

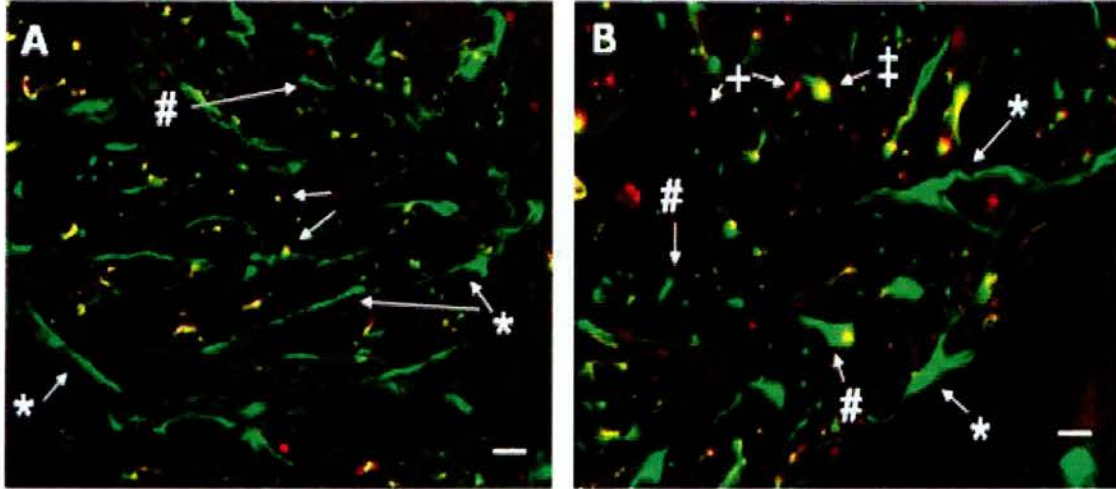


Figure 6: cGMP and nNOS staining in the outer muscle bundles of the base of the guinea pig bladder. cGMP positive cell processes and cells run over the surface of the muscle bundles ((*), SM-ICs). Cells reactive to cGMP can also be seen within the muscles bundles ((#), IM-ICs). As in the previous figures the tissue was incubated in isobutyl-methyl-xanthene, followed by NO_x prior to staining. (This illustration has been reproduced with permission from Professor James Gillespie and BJU International; Gillespie et al, 2004).

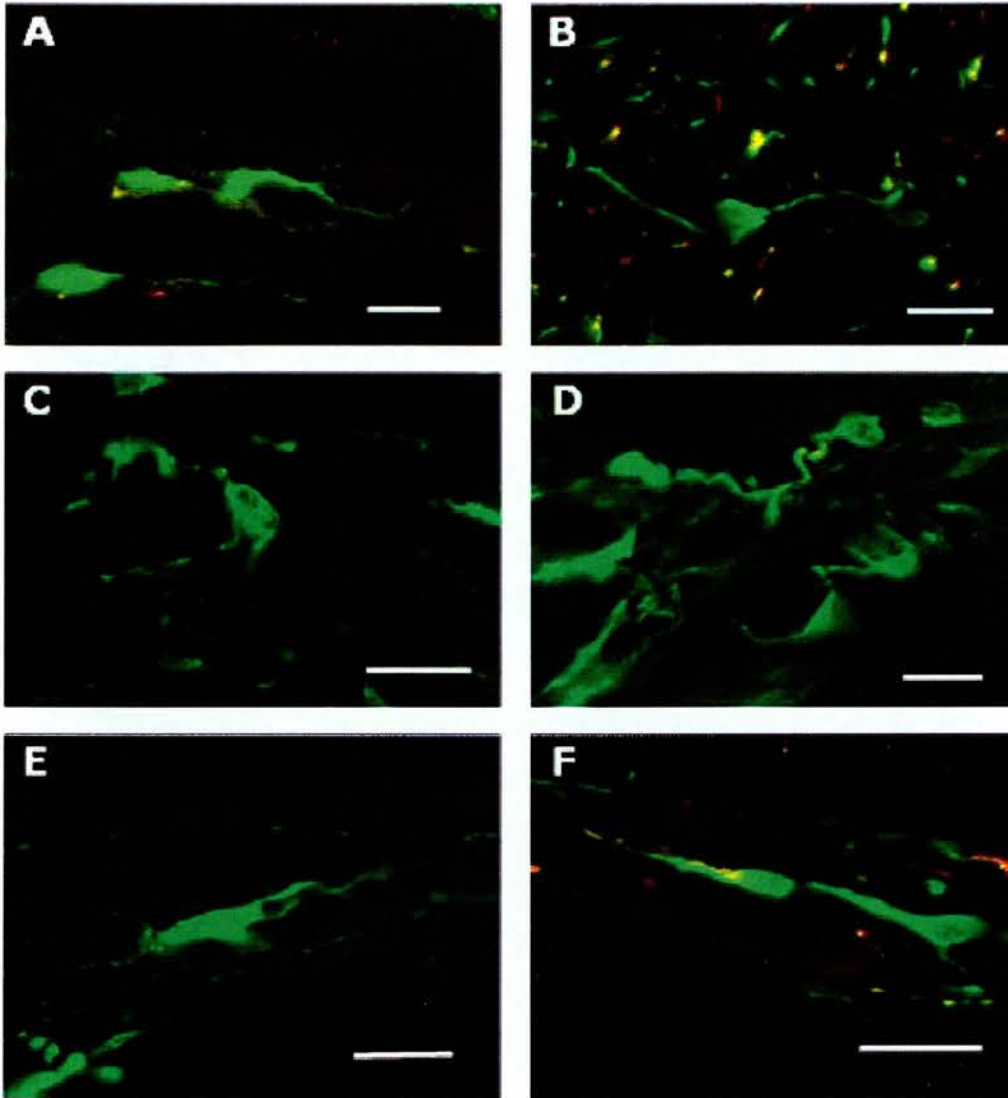


Figure 7: cGMP and nNOS staining of cells within the muscle bundles (IM-ICs). A and B are from the base of the guinea pig bladder, C and D from the lateral wall, and E and F from the dome. The cells have large central cell bodies and processes can be seen running from the cell bodies parallel to the smooth muscle fibres. These cells are found primarily in the central regions of the muscle bundles with only the cell processes apparent at the ends of bundles. Horizontal bar at the bottom right represents 10µm. (This illustration has been reproduced with permission from Professor James Gillespie and BJU International; Gillespie et al, 2005).

1.5.2 Ganglia

Intra-mural ganglia are found in the bladder wall of many species, including man (Smet et al, 1997; Zhou and Ling, 1998; Gabella, 1990). At present there are no data to demonstrate a specific physiological role for this network. The intra-mural ganglia receive inputs from nerve fibres containing calcitonin gene related peptide (CGRP) and substance P (sub P), (Smet et al, 1997; Zhou and Ling, 1998). These fibres are likely to be collaterals from sensory fibres emanating from the sub-urothelial layer. The observation, that CGRP can inhibit muscarinic augmented phasic activity has led to the suggestion that the intra-mural ganglia play a role in the generation or modulation of phasic activity (Gabella, 1990), with the interstitial cells being involved in the distribution of this activity to larger areas of the bladder wall. Examples of ganglia from the bladder wall of the guinea pig are illustrated, figure 8.

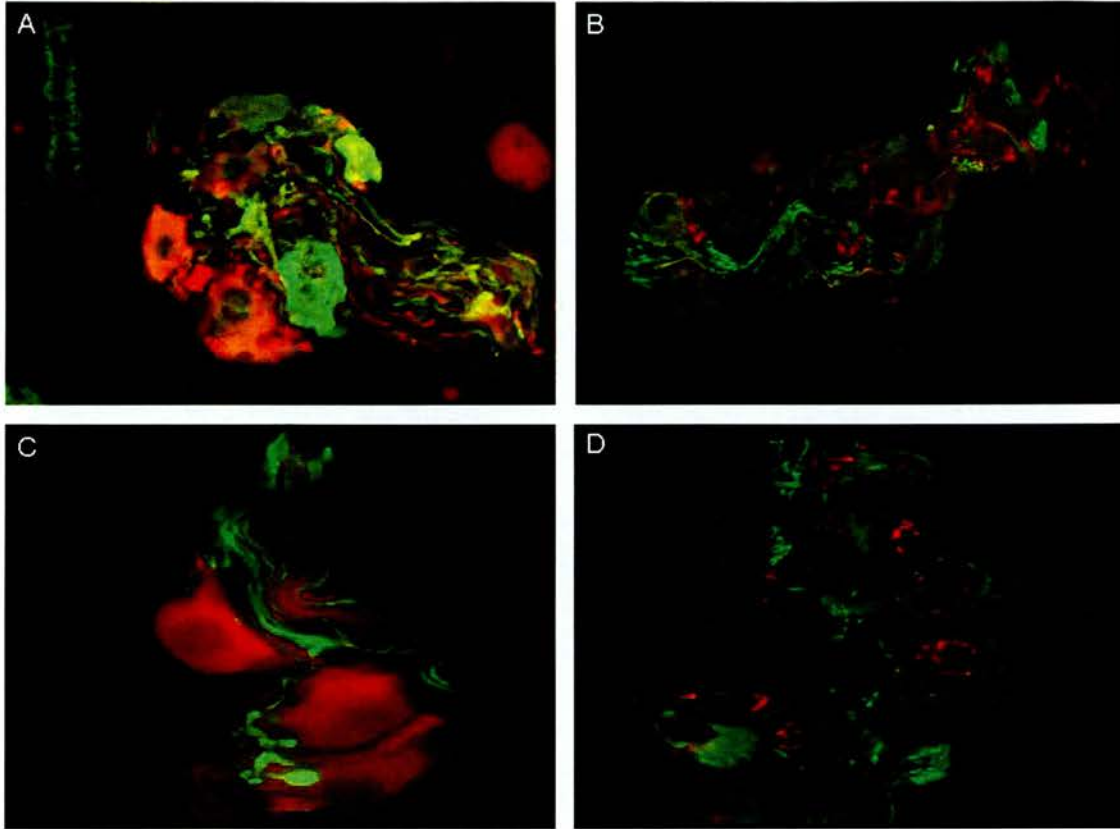


Figure 8: Fluorescent images of ganglia detected within the guinea pig bladder. A represents a ganglion from the outer muscle layer, B – D represent ganglia from the suburothelial layer. In A, cell bodies positive for nNOS (red) and cGMP (green) are seen. B shows a ganglion stained for neurofilament protein (NF), green, and for Choline acetyltransferase (ChAT), red. C shows a suburothelial ganglion staining for calcitonin gene related peptide, green, and ChAT, red. D shows a ganglion staining for α_{1b} adrenergic receptors, red, and NF, green. (These illustrations have been reproduced with kind permission from Professor James Gillespie).

1.6 Autonomous activity: integrated physiology of the isolated guinea pig bladder

As previously mentioned, regular phasic rises in intravesical pressure, similar to non-micturition activity seen 'in vivo', can be seen in the isolated bladder (Sherrington, 1892). More recently these observations were re-examined in the isolated guinea pig bladder (Drake et al, 2003a; Drake et al, 2003b). Spontaneous complex activity was observed on the outer surface of unstimulated bladders. This activity, termed 'autonomous' activity, consisted of localised micro-contractions, waves of activity and microstretches confined to discrete regions of the bladder surface, which were associated with transient rises in intravesical pressure. Incremental increases in intravesical volume recruited additional areas of activity with subsequent increases in phasic intravesical pressure. The addition of atropine or tetrodotoxin did not block this activity suggesting the underlying mechanisms to be unrelated to the neuromuscular junction or intrinsic nerves. The addition of arecaidine, a muscarinic agonist, or the nicotinic ligand Lobeline augments the underlying 'autonomous activity'. Initially an increase in the number of regions displaying micro-contractions is seen followed by the development of large, co-ordinated waves of contraction over the surface of the bladder. These lead to the generation of larger rises in pressure associated with transient contractions with an associated increase in frequency: termed 'augmented' activity, (Drake et al, 2003a). These simple effects have previously been highlighted as important observations as they suggest this activity to be independent of central mediation and generated by mechanisms distinct from post-ganglionic cholinergic activation (Drake et al, 2003c). Additionally, as previously mentioned, in the cat these phasic contractions are associated with bursts of afferent impulses thought to emanate

from rapidly adapting stretch receptors (Andersson and Yoshida, 2003; Vaughan and Satchell, 1995).

1.6.1 Effects of increasing intravesical volume

From these experiments upon the isolated bladder certain characteristics and responses of the local mechanisms within the bladder wall have been well documented. It can be seen that the amplitude and frequency of transient contractions increase with increasing intravesical volume. This has been shown to be the case in both the unaugmented ('autonomous' activity) and augmented state ('augmented' activity). Figure 9 demonstrates the effects of increasing intravesical volume on autonomous activity. In addition, with a fixed intravesical volume increasing doses of muscarinic agonists similarly increases the amplitude and frequency of transients in a fashion similar to increasing the intravesical volume. However, a much more marked effect is seen with the muscarinic agonists, Figure 10, (Drake et al, 2003c).

1.6.2 Effects of nitric oxide

The addition of the nitric oxide (NO) donor sodium nitroprusside (SNP) results in an excitatory response, increasing the frequency of phasic contractions, (Gillespie JJ et al, 2004b). This would suggest that the nitrergic innervation of the bladder wall is associated with an overall excitatory input to the mechanisms involved in the generation of phasic activity. It has been shown that the detrusor smooth muscle does not respond to NO stimulation with a rise in cGMP. However, the cells that do respond in this manner are the suburothelial and intramuscular interstitial cells previously

mentioned (Smet et al, 1996). Therefore, it is likely that interstitial cells are integral to the generation and modulation of phasic activity (Gillespie et al, 2004b).

A small population of nerves within the guinea pig bladder also show elevated levels of cGMP in response to SNP (Smet et al, 1996). A further possibility is that in addition to an action directly upon interstitial cells, the excitatory effects of SNP application on phasic activity could be mediated via these neural mechanisms. The increase in the frequency of phasic activity induced by SNP could arise indirectly by either the stimulation of excitatory neurones or the inhibition of inhibitory intramural neurones (Gillespie et al, 2004b).

1.6.3 Effects of noradrenaline

In contrast to the excitatory effect of muscarinic agonists sympathomimetic stimulation, via the addition of noradrenaline, results primarily in an inhibition of phasic activity. A reduction in the amplitude of each transient is seen, coupled with a reduction in the frequency of phasic activity. Two receptor subtypes have been identified, α_1 and β_3 , that are involved in this inhibition with both displaying dose dependent responses. The presence of these two functionally relevant receptor subtypes suggests that noradrenaline has a number of sites of action within the bladder with the mechanisms generating or modulating phasic activity receiving inputs from adrenergic nerves (Gillespie, 2004c).

1.6.4 Effects of Adenosine Triphosphate

Following mechanical distortion, the urothelium is known to release adenosine triphosphate (ATP), (Fergusson, 1999; Birder et al 1998, 2001, 2002). A non-hydrolysable form of ATP, α/β methylene ATP, was found to have a marked excitatory effect on phasic activity leading to an increase in both frequency and amplitude; in addition to its direct effect on smooth detrusor muscle. Interestingly, following 'washing out' of α/β methylene ATP from the preparation a profound inhibition is seen. It is known that the excitatory effects of ATP are exerted via the P2X receptors (McMurray et al, 1998) and in relation to autonomous activity these seem to be rapid in onset. However, those leading to the resultant inhibition are less rapid with an uncertain site of action (Gillespie, 2004a).

1.6.5 Effects of substance P and calcitonin gene related peptide

Phasic activity has also been found to be modulated by the sensory neuropeptides, substance P and calcitonin gene related peptide (CGRP). Substance P was found to be excitatory and CGRP inhibitory. The addition of capsaicin, known to release both sub P and CGRP from sensory nerves, resulted in an excitation followed by an inhibition. These observations suggest that the mechanisms generating complex phasic activity in the bladder wall have an innervation from collateral axons of sensory nerves; suggesting a local axonal reflex present within the bladder wall, (Gillespie, 2005a).

1.6.6 Intrinsic changes in spontaneous activity during changes in intravesical volume: the volume response

In addition to the effects of specific neurotransmitters known to be present within the bladder wall, the integrated responses of the isolated bladder to changes in volume, similar to those seen during micturition, have been characterised. It can be seen that the frequency and amplitude increases with increased volume. However, following a sudden increase in intravesical volume a rapid increase in frequency and underlying basal pressure is seen. This initial increase in frequency settles and falls to a steady state which is associated with a reduction in underlying basal pressure. Although the frequency and basal pressure are still higher than those seen prior to the volume increase. Once the intravesical volume is reduced back to baseline values a quiescent period is seen. The frequency and amplitude of transient phasic contractions slowly return over a period of time (Lagou, 2005). An example of a typical 'volume response' is shown in Figure 11.

The addition of tetrodotoxin to the isolated bladder or pre-treatment with capsaicin has no effect upon these volume induced responses. This would suggest that since most neural sensory influences would be eliminated by this combination, local sensory collateral reflexes are not directly involved with volume induced responses. However, the effects of ODQ (oxadiazole quinoxalin-1-one) in reducing the volume induced responses would support the involvement of interstitial cells, (Lagou, 2005).

From these experiments on the isolated guinea pig bladder a complex series of responses inherent to mechanisms within the bladder wall can be seen, each with the

potential of modulating phasic activity. This 'motor/sensory' system has the potential to send bursts of afferent activity to the CNS proportional to intravesical volume. The effects of which can be modulated by the neurotransmitters noradrenaline, substance P, calcitonin gene related peptide and nitric oxide; suggesting a role for intramural nerves. The effects of adenosine triphosphate in turn suggest a role for the urothelium. Although the underlying mechanisms are not entirely clear, what is emerging is that these local responses combine to form a modulated sensory system capable of altering afferent discharge in response to both local and central inputs. Due to the relative sensitivity of this system to the effects of acetylcholine it is feasible that it may be a target for antimuscarinics. Additionally, due to the number of inputs to this system it has the potential to be used as a direct target for novel new therapies in the treatment of OAB.

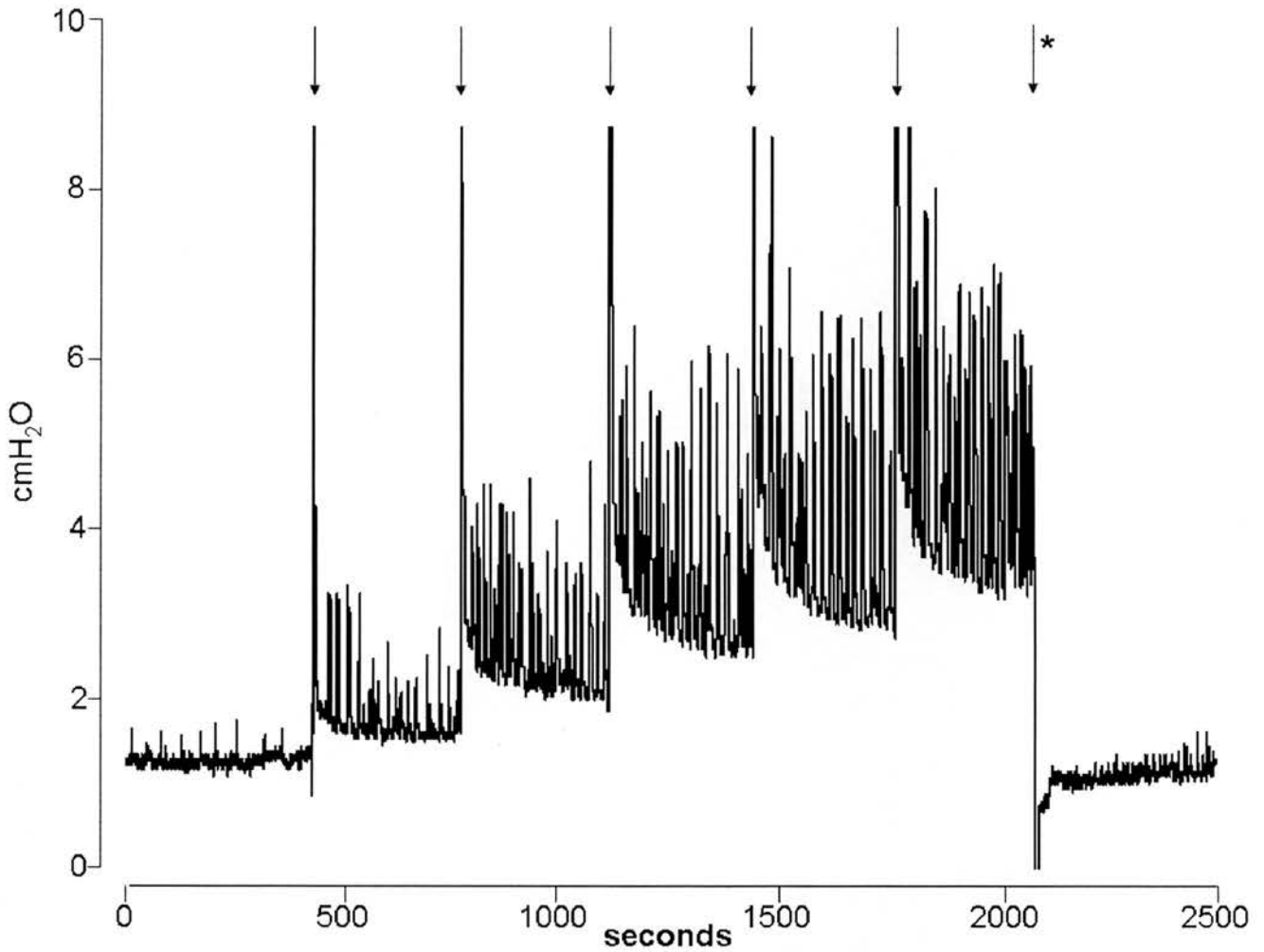


Figure 9: a typical example of the effects of increasing volume upon autonomous activity as described by Drake et al, (2003c). The trace represents intravesical pressure measured in an isolated guinea pig bladder, (taken from the author's own personal records). At time point 0 seconds the intravesical volume is at baseline volume, 800 μ l. Each arrow (\downarrow) represents an incremental increase in intravesical volume of 400 μ l, typically taking 3 seconds to be instilled. (\downarrow^*) represents a reduction in volume of 2000 μ l. It can be seen that with increasing volume both the frequency and amplitude the transient contractions increases. Ordinate axis represents pressure (cmH₂O), and abscissa time (seconds).

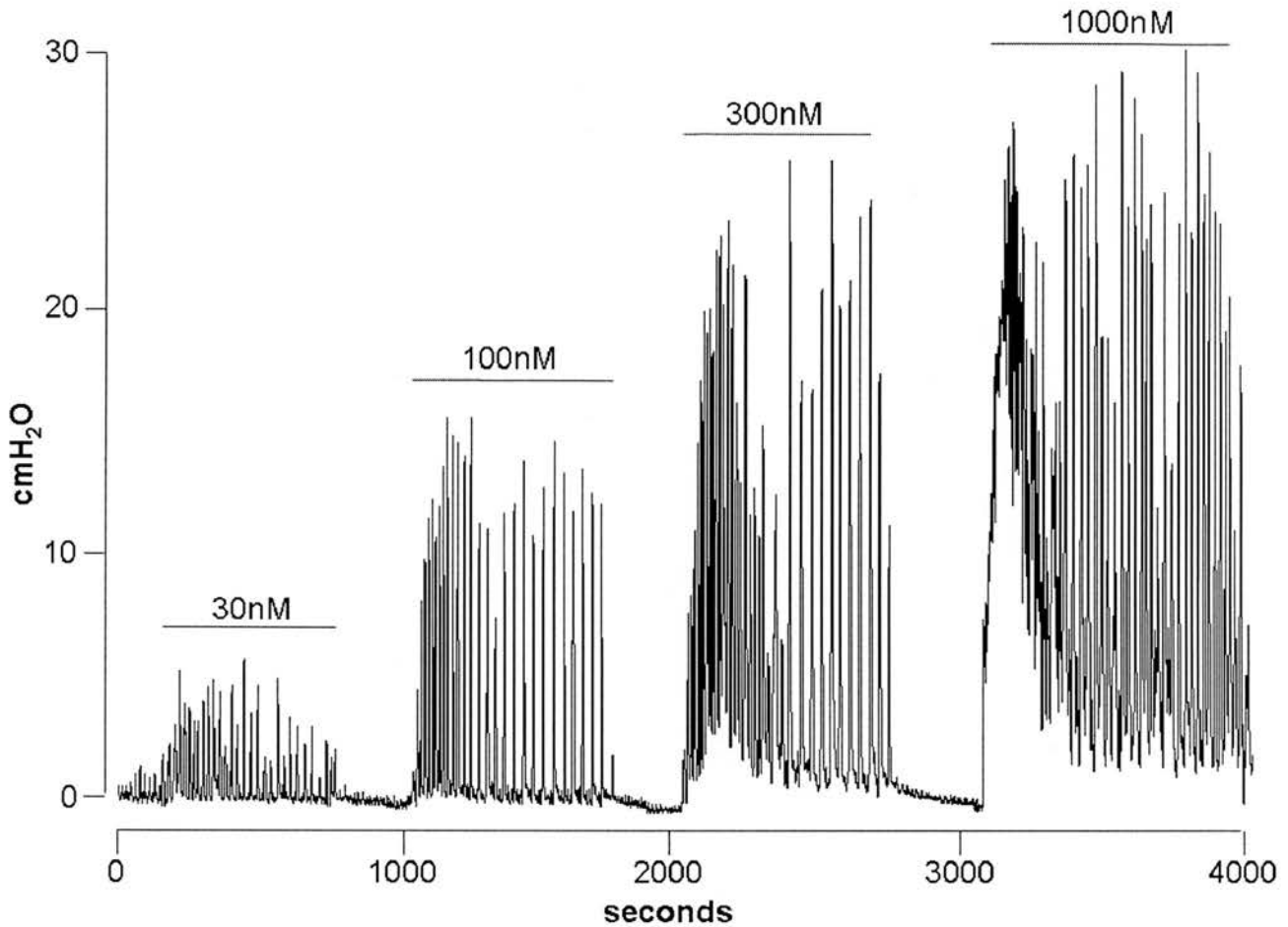


Figure 10: a typical example demonstrating the effects of increasing doses of muscarinic agonist, arecaidine, upon 'autonomous activity' as previously described by Drake et al, (2003c); the trace is taken from the author's/ candidates own records. Arecaidine was added at the concentration shown during the time indicated by the horizontal bar. Between drug applications the bladder was washed in Tyrode's solution. During the 'washes' and prior to the addition of agonist small regular phasic rises in intravesical pressure can be seen; termed 'autonomous activity'. Following addition of agonist an increase in the frequency and amplitude of transient contractions is seen. This activity, in the presence of muscarinic agonist, is termed 'augmented activity'. Ordinate axis represents pressure (cmH₂O), and abscissa time (seconds).

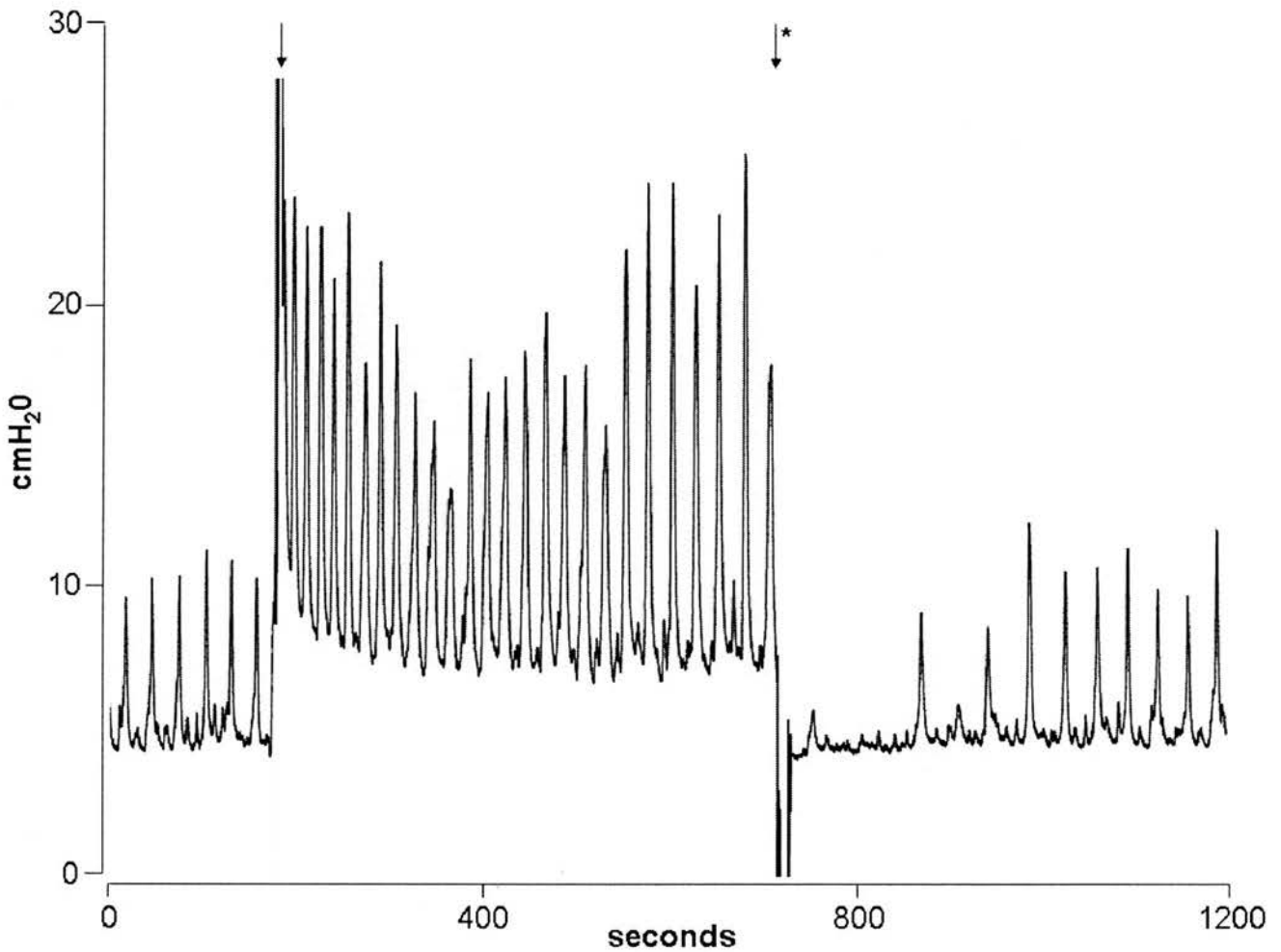


Figure 11: demonstrating a typical 'volume response' performed on an isolated guinea pig bladder, with phasic activity augmented by 100nM arecaidine, as described by Lagou et al, (2005); trace taken from the author's own records. (↓) represents a 2000μl volume increase and (↓*) a 2000μl decrease. Following the volume increase an increase in frequency can be seen that settles down to a 'steady state'. Following a volume decrease a quiescent period can be seen with the frequency and amplitude of transient contractions slowly returning. Ordinate axis represents pressure (cmH₂O), and abscissa time (seconds).

1.6.7 Overview of the human clinical relevance of observations of autonomous activity in animal models.

As previously discussed, autonomous activity has been documented in a number of animal models; cat, mouse, guinea pig, rat, monkey and dog. Although its exact function isn't fully known, in the cat each transient contraction has been documented to be associated with a burst of afferent activity, (Vaughan and Satchell, 1992). Since the frequency of autonomous activity is associated with bladder volume it is therefore assumed that autonomous activity is related to the perception of intravesical volume. Support for this idea is seen in other animal models, where autonomous activity has been found to be related to micturition frequency (Streng et al, 2006). In these cases, increased autonomous activity is associated with an increase in the frequency of micturition and a subsequent reduction in bladder capacity.

If such a system were to exist in the human then autonomous activity may act as a means of mechanical-neurochemical transduction whereby intravesical volume can be determined. As such it may play a role in the perception of bladder sensation. Abnormalities of this system may also be involved in the origin of increased micturition frequency and reduced bladder capacity, and/ or, the development of the symptom of urgency. Although no experiments have been performed as of yet to solely look at autonomous activity in the bladder, indirect evidence for the presence of bladder contractions unrelated to micturition have been seen on standard and ambulatory urodynamic assessments (Robertson et al, 1994; Heslington et al, 1996). Work described later in the thesis describes how autonomous activity is sensitive to the effects of muscarinic agonists/ antagonists, and it is postulated that autonomous activity may act as means through which anti-muscarinic medications exert their beneficial effects.

The experiments upon animal models described above are largely limited since only objective measurements indirectly associated with sensation (micturition frequency and bladder capacity) can be made of animal voiding patterns. They do not allow an appreciation of what the animal is actually thinking or their perception of bladder sensation. In the human, however, the reverse is true. Animal experiments leading to the above ideas are generally irreproducible in humans as it is difficult to obtain normal healthy human bladders for isolated experiments. In addition it would also be difficult to perform sensory afferent recordings. However, the advantage of the human over the animal models is that the human is able to allow both the measurement of objective recording of voiding patterns in addition to vocalising the rather more subjective assessment of bladder sensation. Therefore, to fully translate the relevance of autonomous activity to the human a combination of animal and human work needs to be performed.

From each individual animal model it is possible to obtain data relating to distinct physiological phenomenon and attempt to translate this to specific structures within the bladder wall. Although difficult to achieve solely from a single animal model if studies are repeated in a number of animal models, differences in physiology may be attributed to structural variations, allowing some degree of assumption to be made regarding the role of certain structures. Attempts can then be made to relate structure to function. Certainly this has been seen already when traces from guinea pig bladders are compared to those of the mouse. A distinct difference is seen in the character of these traces, with mouse tracings being more sinusoidal in nature actively contracting above and relaxing from a common basal pressure, (Lagou et al, 2004). Whereas guinea pig traces tend to peak from an underlying basal pressure, (Figure 10), suggesting active contraction with

little in the way of active/ passive relaxation below basal pressure. Since the mouse bladder has no suburothelial interstitial cells, a putative suggestion is that the suburothelial interstitial cell layer contributes to maintaining this underlying basal tone. Although by no means complete, this comparison illustrates the nature of relating structure to function and if performed on a number of animal models more accurate assumptions may be able to be formulated for certain structures. This thesis concentrates solely upon phenomenon characterised in the guinea pig bladder, as such a degree of caution needs to be exercised in the translation of this work into the human model.

Though it is extremely difficult to reproduce experiments on isolated human bladders, access to human bladder tissue is a lot less problematic. It is therefore possible to harvest normal bladder tissue at the time of operations, ethics permitting, to examine its ultra-structure. Once more is known about the structure of the bladder then attempts can be made to relate this to its function. This can also be repeated for pathologically diseased bladders. In addition to this approach, iso-volumetric urodynamics may also be performed on the human (in a fashion similar to standard urodynamics) where autonomous activity is correlated both to intravesical volume and to sensation, and also to structure. Once more is known about the relevance of autonomous activity to sensation, both in the normal and pathological states, pharmacological manipulations and subsequent treatments may then be developed.

1.7 The Overactive Bladder syndrome (OAB): a clinical perspective and role of antimuscarinic medications

1.7.1 Basic Problem

The overactive bladder syndrome (OAB) is a symptom complex characterised by urgency, with or without urge incontinence, usually accompanied by frequency and nocturia (Abrams et al, 2002). It is a common condition affecting 17% of the populations of Europe and the USA (Milsom et al, 2001; Stewart et al, 2001). But despite its prevalence the aetiology of OAB is not fully understood. Possible explanations as to the aetiology of OAB have been proposed which, to a certain extent, go some way in explaining a number of the observations seen. Examples of which include the neurogenic hypothesis, abnormal expression of the micturition reflex (de Groat, 1997), and the myogenic hypothesis, changes in the properties of the detrusor smooth muscle (Brading et al, 1994). However, no single hypothesis is, as yet, able to fully incorporate all the observations seen in both the normal and abnormal states.

Although not fully understood, the symptoms of OAB have been generally considered to be due to abnormal bladder contractions occurring inappropriately during the filling phase. On this assumption the mainstay of treatment for OAB has been anti-cholinergic medications, in the belief that they will reduce the contractility of the detrusor muscle. The logical argument was that by preventing these abnormal contractions this would relieve symptoms. There is no doubt that the antimuscarinic drugs currently used in clinical practice (oxybutynin, tolterodine, trospium, solifenacin and darifenacin) are effective in reducing the symptoms of OAB. Also, there is no doubt, that at high concentrations, antimuscarinics can reduce the amplitude of detrusor contraction.

Indeed, patients given an excess of antimuscarinic drugs retain urine due to an inability of the bladder to contract (Stahl et al, 1995).

Despite a large number of studies demonstrating the effectiveness of antimuscarinics on symptoms of OAB there are few studies examining their actions on the bladder at the doses which are effective on OAB symptoms. Certainly, little data is available on whether antimuscarinics actually reduce the strength of abnormal contractions, thus answering the question; 'Are the beneficial effects of antimuscarinics due to a reduction of the DO component of OAB?' Despite this lack of data there is a slow but growing anecdotal realisation that, at clinically effective doses, the drugs have little effect on bladder contractility. If this is so, the generally accepted view of their mode of action is incorrect.

1.7.2 Further complications

Only 54% of patients presenting with the OAB symptom complex show involuntary bladder contractions during a cystometric investigation (Digesu et al, 2003). These patients subsequently respond well to antimuscarinic drugs and the symptoms of OAB decrease. Thus, at first sight, it would appear that this group of patients behave according to 'classical theory'. In the remaining 46% of patients however, bladder contractions are not seen during filling (Digesu et al, 2003). These patients have what has previously been referred to as 'sensory urgency' as opposed to detrusor over activity. Interestingly, these patients with OAB but no DO appear to respond equally well to the antimuscarinic drugs with reduced symptoms of urge and incontinence (Hashimoto et al, 1999). Based on the 'classical theory' there is no reason why these

patients should benefit from an antimuscarinic drug since they have no pathological contractions of the detrusor to inhibit.

A further complicating factor comes from studies of healthy asymptomatic volunteers undergoing conventional urodynamics and ambulatory urodynamics. In one study 17% had activity during conventional urodynamics similar to that seen in patients with OAB. During ambulatory urodynamics 38% of these normal subjects had contractions during the filling phase of the micturition cycle (Robertson et al, 1994). In a different study 60% of asymptomatic normal volunteers had bladder contractions during the filling phase (Heslington et al, 1996). These observations bring into question the significance of the apparent correlation of bladder contractions with OAB. If a high proportion of 'normal' subjects have bladder activity during filling, is it pathological or physiological?

These observations therefore present three fundamental problems:

Why are the antimuscarinic drugs effective in patients with OAB but no DO? Why do only half of the patients with OAB symptoms have DO?

Why do 60% of asymptomatic normal individuals have bladder activity during filling?

1.7.3 Effects of antimuscarinics upon muscle strips

The role of antimuscarinic drugs upon cholinergic mediated contraction of the detrusor muscle is well established. For example, oxybutynin reduces carbachol induced contractions in isolated animal bladder strips (Waldeck et al, 1997; Tonini et al, 1987; Yono et al, 1999). It also reduces electrically stimulated contractions in guinea pig and human detrusor strips (Waldeck et al, 1997; Tonini et al, 1987; Yono et al, 1999). Similarly, tolterodine has been shown to reduce carbachol induced contraction of human detrusor strips (Yono et al, 1999). Therefore, there can be no doubt that inhibition of muscarinic receptors on the smooth muscle can decrease the force of agonist or nerve mediated cholinergic contraction.

1.7.4 Role of muscarinic receptors

The receptors involved are muscarinic receptors. Five distinct subtypes of muscarinic receptor have been described, M_1 – M_5 (Caulfield et al, 1998). All five receptor subtypes are found in the bladder but the most abundant are the M_2 and M_3 subtypes. In the human bladder the M_2 receptors predominate (2/3) and are more abundant than M_3 (1/3), (Andersson and Wein, 2004). Data from knock out mice show clearly that the M_3 receptors are responsible for activating the contraction of the detrusor (Hegde et al, 1999; Chess-Williams et al, 2001; Fetscher et al, 2002; Yamanishi et al, 2001). For this reason M_3 specific drugs were developed to treat bladder over activity. The function and role of the M_2 receptors is not known. However, there is the suggestion that M_2 receptors may oppose β -adrenoreceptor mediated sympathetic relaxation of the detrusor via the inhibition of adenylyl cyclase (Hegde et al, 1997; Yamanishi et al, 2002). Muscarinic receptors are also found on bladder urothelial cells and on structures in the

suburothelium (interstitial cells, nerves), (Mansfield et al, 2005). Therefore antimuscarinics have the capacity to block muscarinic receptors elsewhere and not just on the detrusor muscle where they decrease the ability of the bladder to contract. However, clinically, antimuscarinics act mainly during the storage phase, decreasing urge and increasing bladder capacity.

1.7.5 In vivo animal models of bladder dysfunction

It has proven possible to make animal models of OAB where micturition frequency is increased and the animals may also leak urine. When the antimuscarinic drugs are studied on these models it is clear that they decrease the frequency of voiding and increase bladder capacity. However, at these therapeutic doses little effect was noted on the ability of the bladder to contract. For example, in conscious rats, with an increased frequency of micturition due to urethral obstruction, oxybutynin (1mg/kg iv) decreased frequency but did not significantly reduce micturition pressure (Guarneri et al, 1991). Similarly, doses of solifenacin and tolterodine, sufficient to significantly raise the bladder capacity of conscious rats with OAB induced by cerebral infraction, did not significantly reduce micturition pressure (Suzuki et al, 2005).

Further supporting an effect of antimuscarinics on afferent nerves, low doses of intravenous tolterodine were also found to significantly increase bladder capacity in vehicle treated rats with detrusor overactivity caused by cerebral infarction, but had no effects on bladder capacity in rats treated with resiniferatoxin, (Yokoyama et al, 2005). Also, intravesical administration of tolterodine significantly increased bladder capacity in control rats with cerebral infarction, but had no effect on bladder capacity in

resiniferatoxin treated animals. Yokoyama et al therefore suggested that at low doses tolterodine exerts an inhibitory effect on C-fibre bladder afferent nerves, thereby improving bladder capacity during the storage phase.

These animal studies clearly illustrate the basic problem. At high doses the antimuscarinic drugs have a direct effect on muscarinic receptors situated on the detrusor muscle, preventing agonist or nerve mediated contractile responses. However, at lower doses, where they clearly affect micturition frequency or bladder capacity, they have little to no effect on contraction. The conclusion must be that inhibition of bladder function by M₃ specific antimuscarinic drugs must occur at receptors other than those on the detrusor smooth muscle which are responsible for contraction.

1.7.6 In vivo evidence from human studies

An important question is therefore *‘does this discrepancy occur in humans’?* There have been very few studies which have examined or reported parameters associated with bladder contractility in patients with detrusor overactivity before and after treatment with antimuscarinic drugs. This is somewhat surprising given the prevalence of the syndrome and the necessity of understanding how the drugs work.

Therefore in order to investigate the cystometric effects of antimuscarinics at clinically effective doses the available literature was reviewed, (Finney et al, 2006). Using a standard ‘pubmed’ electronic search only 14 original articles (including 2 abstracts) containing cystometric data for both filling and voiding phases in patients with DO before and after treatment were identified. In these articles a great deal of variation in

the cystometric parameters reported, patient numbers and methods of analysis was seen. These factors made a formal cumulative statistical analysis difficult. In addition, the changing definition of an overactive contraction during cystometry, from those restricted to over 15cmH₂O to all clinically relevant involuntary rises in detrusor pressure, made a cumulative comparison inappropriate as a further subdivision in data would occur. Therefore, due to the limitations in this approach, data from each individual article was deemed to be significant, based upon $p < 0.05$, and descriptive analysis used to compare results between articles.

For the purpose of this analysis the published articles were divided into three groups dependent upon the patient group described; “neuropaths”, “idiopaths” and mixed (or not specifically stated), labelled Groups I-III respectively. In Group I (neuropaths; table one), data was reviewed from three articles (Madersbacher et al, 1995; Goessl et al, 2000; Stöhrer et al, 1991) one of which contained two treatment groups (Madersbacher et al, 1995), making a total of four reported treatment arms. During filling all treatment arms commented upon an increase in maximum cystometric capacity (C_{max}), with two arms both showing a significant decrease in maximum detrusor pressure (p_{detmaxfilling}). The effects on compliance were equivocal; two arms showing no significant change and two a significant increase. In reported voiding parameters, maximum pressure (p_{detmax}) was significantly decreased in two arms, both from the same article. No significant change was seen in maximum flow (Q_{max}) and the effects on residual volume were equivocal.

In group II (idiopaths; table two), data was reviewed from three articles and one abstract (Giannitsas et al, 2004; Madersbacher et al, 1999; Athanasopoulos et al, 2003; Rosario et al, 1996). One article, the only article to do so, subdivided patients with OAB into four categories dependent on the volume and magnitude of the first overactive contraction (Giannitsas et al, 2004). Patients were labelled as high volume/low pressure, high volume/ high pressure, low volume/ low pressure or low volume/ high pressure dependent on the volume and pressure of the first overactive contraction with respect to the values 250mls and 25cmH₂O respectively. This subdivision was performed in the assessment of two antimuscarinics resulting in 8 treatment arms. However, as two of these arms had insufficient numbers only six from this article were available for analysis, with one each from the remaining three.

In group II the effects of antimuscarinics on parameters associated with the storage phase were not clear cut. First desire to void (FDV) was significantly increased in five treatment arms with two reporting no significant change. This is mirrored by the volume at first overactive contraction (VFOC), with six showing a significant increase and two no change. Interestingly, in group II, maximum cystometric capacity (C_{max}) was only significantly increased in three of the arms, and not significantly changed in five, conflicting with data from Group I. The maximum pressure of the largest overactive contraction (p_{detmax-oc}) again was equivocal with one arm showing a significant decrease and one no significant change. However, in all groups reporting the pressure of the first overactive contraction (PFOC), there was no significant change.

The effects on parameters associated with the voiding phase were unequivocal, although relatively few arms were available for comment. No significant changes were seen in either maximum pressure during micturition (pdetmax), maximum flow (Qmax) or residual volume.

In group III (mixed or aetiology not specified; table three), data was reviewed from six articles (Jonas et al, 1997; Cardozo et al, 2000; Thüroff et al, 1991; Wagg et al, 2003; Goode et al, 2002; Griffiths et al, 1994) and one abstract (Abrams et al, 2002). Group III contained a combination of mixed patient groups and groups where aetiology was not commented upon. Each article contained one treatment arm examining the effect of an antimuscarinic in current routine use. (One treatment arm containing data for an older medication, propantheline, was not analysed). In one article (Goode et al, 2002), anti-cholinergic and behavioural modification therapy was compared solely with behavioural modification. Although the results for the effects of antimuscarinics were not directly presented, they could be deduced from the results.

In this mixed group, antimuscarinics significantly increased both the volume at which the first overactive contraction occurs (VFOC) and the maximum cystometric capacity (Ccm_{ax}), suggested by four and six articles respectively. In addition, the pressure of first overactive contraction (PFOC) and the maximum pressure of the largest overactive contraction (pdetmax-oc) were not significantly changed; although only one article apiece described these findings.

No conflict was seen in voiding parameters. Three articles reported no significant change in maximum flow (Q_{max}) or detrusor pressure at maximum flow ($p_{det}Q_{max}$). Six articles suggested an increase in residual volume, and in one there was no significant change in detrusor pressure at either urethral opening or closure.

In general a uniform trend can be seen between groups II and III. However, the main exception to an overall trend is the effect on reported detrusor pressures seen in group I (neuropaths); with two arms from the same article demonstrating a significant decrease in detrusor pressure during both filling and emptying. This may be representative of the small numbers involved, or, signify difference in the aetiologies of “idiopathic” and “neuropathic” OAB.

In examining the data as a whole a more convincing picture emerges as to the effects of anti-cholinergics on urodynamic findings. Parameters associated with storage, such as first desire to void (FDV) and maximum cystometric capacity (C_{cmax}) are significantly increased in the vast majority of articles. With parameters associated with voiding, such as the pressure at maximum flow ($p_{det}Q_{max}$) and maximum flow (Q_{max}) not being significantly altered. However, the effect on $p_{det}max$ seems equivocal, with two articles suggesting a decrease in pressure and one suggesting no significant change.

Descriptive analysis of the data allows some conclusions to be formulated for the majority of urodynamic parameters as either the results are consistent between articles, or in the case of some disparity, the vast majority of articles suggest one variable over another. In cases of equivocal results, articles were torn between either similar changes

or no change. There were no cases of opposing results between articles, i.e. a decrease and an increase.

This analysis illustrates an absence of data to support antimuscarinics having a significant inhibitory effect upon detrusor contraction. Most notably, no effect was seen in the idiopathic (or 'mixed') group of patients and although some reduction was seen in the neuropathic group, this was limited to one article. This anomaly with the neuropathic group may represent the small number of articles involved overall. Or alternatively, be representative of potential differences behind the aetiologies of 'idiopathic' and 'neuropathic' detrusor overactivity. Especially since the majority of neuropaths generally display higher detrusor pressures, which may be more sensitive to effects of anti-cholinergics.

It must be emphasised that these findings do not dispute the effects of anti-cholinergics at higher doses but do suggest that at clinically relevant doses alternative effects need to be considered. The benefits of decreased urgency, frequency and leakage arise through improvements in bladder capacity and sensations to void; not, in the case of idiopaths, through reducing the ability of the detrusor to contract. Consequently, it must therefore be assumed that antimuscarinics act at sites other than at the detrusor, effecting alternative cholinergic mechanisms involved in either the generation or modulation of sensation.

Reference	Madersbacher et al (1995)	Madersbacher et al (1995)	Goessl et al (2000)	Stöhrer et al (1991)
Drug	Trosp.	Oxy.	Tolt.	Trosp.
Dose	20mg bd	5mg tds	0.1mg/ kg	20mg bd
Number (n)	52	43	12	29
C_cmax (ml)	inc.	inc.	inc.	inc.
Compliance (ml/cmH₂O)	no change	no change	inc.	inc.
pdetmax (filling) (cmH₂O)	-	-	dec	dec
pdet max (cmH₂O)	dec	dec	-	-
Qmax (ml/s)	-	-	-	no change
Residual vol.(ml)	inc.	inc.	-	no change

Table 1: Table showing the effects of antimuscarinics upon cystometric parameters in patients with ‘neuropathic’ related detrusor overactivity. Each column represents a treatment arm whose effects have been compared to placebo. Cystometric parameters are shown on the left. Entries show either a significant increase (inc.), decrease (dec.), or no significant change (no change) compared to placebo ($p < 0.05$) for each of the cystometric variables shown. (-) appears against parameters not recorded in that article.

Reference	Giannitsas et al (2004)	Giannitsas et al (2004)	Giannitsas et al (2004)	Giannitsas et al (2004)	Giannitsas et al (2004)	Giannitsas et al (2004)	Madersbacher et al (1999)	Athanasopoulos et al (2003)	Rosario et al (1996)
Drug	Oxy	Oxy	Oxy	Tolt	Tolt	Tolt	Oxy	Tolt	Darif
Dose	5mg tds	5mg tds	5mg tds	2mg bd	2mg bd	2mg bd	5mg bd	2mg bd	2.5mg/ 10mg stat
Comment	G2	G3	G4	G2	G3	G4			
Number (n)	25	36	40	25	36	40	145	25	18
FDV (ml)	inc.	inc.	inc.	no change	inc.	no change	inc.	-	-
VFOC (ml)	inc.	inc.	no change	no change	inc.	inc.	-	inc.	inc.
PFOC (cmH₂O)	no change	no change	no change	no change	no change	no change	-	-	no change
pdetmax-oc (cmH₂O)	-	-	-	-	-	-	-	dec.	no change
C_cmax (ml)	no change	no change	inc.	no change	no change	no change	inc.	inc.	-
pdetmax (cmH₂O)	-	-	-	-	-	-	-	no change	-
Qmax (ml/sec)	-	-	-	-	-	-	-	no change	-
Residual vol(ml)	-	-	-	-	-	-	no change	no change	-

Table 2: Table showing the effects of antimuscarinics upon cystometric parameters in patients with 'idiopathic' related detrusor overactivity. Each column represents a treatment arm whose effects have been compared to placebo. Cystometric parameters are shown on the left. Entries show either a significant increase (inc.), decrease (dec.), or no significant change (no change) compared to placebo ($p < 0.05$) for each of the cystometric variables shown. (-) appears against parameters not recorded in that article.

Reference	Jonas et al (1997)	Cardozo et al (2000)	Thüroff et al (1991)	Wagg et al (2003)	Goode et al (2002)	Griffiths et al (1994)	Abrams et al (2002)
Drug	Tolt	Trosp	Oxy	Tolt	Oxy	Oxy	Tolt
Dose	2mg bd	20mg bd	5mg tds	2mg bd	indiv. titrated	2.5 -5mg bd	2mg bd
Number (n)	99	132	63	61	105	59	149
FDV (ml)	inc	-	-	-	no change	-	-
SDV (ml)	-	-	-	-	inc.	-	-
VFOC (ml)	inc.	inc.	inc.	-	-	-	inc.
PFOC (cmH₂O)	-	no change	-	-	-	-	-
pdetmax-oc (cmH₂O)	no change	-	-	-	-	-	-
C_cmax (ml)	inc.	inc.	inc.	inc.	inc.	-	inc.
Compliance (ml/cmH₂O)	inc.	no change	-	-	-	-	-
pdetQmax (cmH₂O)	-	-	-	no change	-	no change	no change
Qmax (ml/sec)	no change	-	-	-	-	no change	no change
Residual vol(ml)	inc.	inc.	inc.	inc.	-	inc.	inc.
pdet-u0 (cmH₂O)	-	-	-	no change	-	-	
pdet-uc (cmH₂O)	-	-	-	no change	-	-	

Table 3: Table showing the effects of antimuscarinics upon cystometric parameters in patients whose aetiology was not stated. Each column represents a treatment arm whose effects have been compared to placebo. Cystometric parameters are shown on the left. Entries show either a significant increase (inc.), decrease (dec.), or no significant change (no change) compared to placebo ($p < 0.05$) for each of the cystometric variables shown. (-) appears against parameters not recorded in that article.

1.8 Summary

The data presented thus far highlights the current inconsistencies in the traditional views of bladder function and patho-physiology. It is emerging that the primary event in the generation of overactive bladder syndrome (OAB) is urgency and this is distinct from detrusor overactivity (DO). Furthermore, the role of DO is becoming uncertain as contractions unrelated to either the act of micturition or the perception of sensation are present in 'normal' healthy individuals as the bladder fills. These contractions are analogous to the 'non-micturition' activity seen in animals and are becoming more apparent with the advent of ambulatory urodynamics. Contrary to traditional thinking antimuscarinic drugs are effective in individuals with OAB but no DO and have been found to exert their beneficial effects through improvements in sensory parameters as opposed to affecting motor activity; suggesting the presence of cholinergic mechanisms involved in the generation/ modulation of afferent activity.

Additional mechanisms involved in the generation of afferent activity have also been demonstrated in addition to the identification of structures with the potential to support these mechanisms. In the cat, regular phasic contractions termed 'non-micturition' activity are associated with synchronous 'bursts' of afferent activity. This activity can be modulated by direct input from the CNS suggesting the presence of an integrated motor/ sensory system. In the isolated bladder this 'non-micturition' activity has been explored further where it has been termed 'autonomous activity'. It has been shown to be sensitive to the effects of both muscarinic agonists and antagonists. It is relatively sensitive to the effects of muscarinic agonists, more so than that of detrusor contraction, and as such may act as a target for current antimuscarinic drugs. This effect would

explain the current clinical anomaly of antimuscarinics effecting sensation as opposed to contraction.

The responses of 'autonomous activity' within the isolated guinea pig bladder have been well characterised, however, the majority of these were studied at fixed intravesical volumes. Where the effects of changing intravesical volume were studied a complex series of events were noted, consisting of a rapid excitation of activity following volume increase, followed by the formation of a steady state with a period of quiescence seen following volume reduction. It is likely that each of these distinct phases are regulated by specific mechanisms within the bladder wall interrelated to form a dynamic process modulating afferent activity during the micturition cycle.

1.9 Experimental Hypothesis

The medical literature suggests that the anti-muscarinic medications currently used in routine medical practise exert their beneficial effects upon sensory parameters, not those associated with motor function. This is at odds with our traditional understanding of both bladder physiology and our treatment of OAB. However, it does suggest that sensory mechanisms exist within the human bladder that are more sensitive to the effects of antimuscarinic medication than motor mechanisms. The targets whereby antimuscarinics may be acting are not clear, however, recent data both relating to bladder structure and physiology has emerged suggesting potential new targets. One of these is the concept of autonomous activity, as discussed previously, in which local reflexes inherent to the bladder wall regulate rhythmical, transient contractions in response to changes in intravesical volume; suggesting a direct motor/ sensory interaction.

The aim of this thesis is to test the hypothesis that autonomous activity is a mechanism through which anti-muscarinic medication may act and to characterise in more detail the phenomenon of autonomous activity. Secondary, experimental aims will be tested to elicit whether other mechanisms are present that alter autonomous activity suggesting the presence of other, potentially therapeutic targets.

To test the hypothesis that autonomous activity represents a sensitive cholinergic mechanism through which anti-muscarinics may act, the effects of increasing doses of muscarinic agonists upon autonomous activity will be assessed (Chapter 3.3) in addition to the effects of increasing doses muscarinic antagonists (Chapter 3.4). This will give some insight into the doses required of both agonists/ antagonists in order to

significantly alter autonomous activity. An idea will therefore be gained as to the sensitivity of cholinergic mechanisms responsible for autonomous activity. In addition, the use of two different agonists and antagonists each with a relative specificity for either M₂ or M₃ muscarinic receptors will allow some insight into the roles of the two muscarinic receptors in the generation of autonomous activity and whether any of the subtypes can be implicated as the therapeutic target of antimuscarinics.

Since the process of micturition is a dynamic process, consisting of a filling and a voiding phase, the response of autonomous activity to increases and decreases in intravesical volume (termed the 'volume response') will be assessed. The work of Lagou et al, 2004 will be expanded to determine the roles of changes in intravesical volume (3.5.3), the duration of volume increase (3.5.4) and the effects of agonist concentration (3.5.5) in the generation of volume induced activity. This will allow further insight into the mechanisms involved in the regulation of autonomous activity in response to volume changes.

It is known that autonomous activity can be modulated by other compounds suggesting further non-cholinergic mechanisms to be also involved in its regulation. Since autonomous activity was found to be sensitive to the effects of muscarinic agonists and antagonists (3.3 and 3.4), especially for those specific to the M₃ muscarinic receptor subtype, it is possible that other mechanisms exist that may subsequently be targeted therapeutically. To explore this idea further, the effects of an anti-muscarinic agent used in current clinical practise (Tolterodine) upon volume induced autonomous activity was tested (3.6) in order to demonstrate what may be happening *in vivo*, bearing in mind the limitations discussed previously, 1.6.7. Subsequently, similar experiments were

performed using nicotinic and purinergic compounds to explore the role of other mechanisms.

The human bladder is known to contain both ganglia and nicotinic receptors, and the aim of these experiments was to examine the role of these in the modulation of volume induced activity. To explore the potential effects of ganglia the effects of increasing doses of the nicotinic receptor antagonist Hexamethonium was assessed, 3.7.1. Hexamethonium was used as this is typically specific to nicotinic receptors associated with ganglia found within the autonomic system. Pancuronium was used to explore the potential for other nicotinic receptor subtypes within this system, 3.7.2. Pancuronium was chosen as this should have no effect upon receptors found upon ganglia.

The role of the purinergic system within the regulation of volume induced autonomous activity was also explored. The aims of these experiments were to assess the effects of purinergic stimulation, non-specific purinergic inhibition and purinergic inhibition specific to P2X₁ and P2Y₁ receptors. To assess the effects of purinergic stimulation the response of volume induced autonomous activity to increasing doses of the purinergic agonist α/β methyl-ATP were recorded, 3.8. The effects of non-specific purinergic inhibition were assessed by the responses to increasing doses of the non-specific purinergic antagonist, PPADS, 3.9. The role of P2X₁ and P2Y₁ purinergic receptors in the modulation of volume induced autonomous activity were assessed by using increasing doses of NF 279 and MRS 2179 respectively.

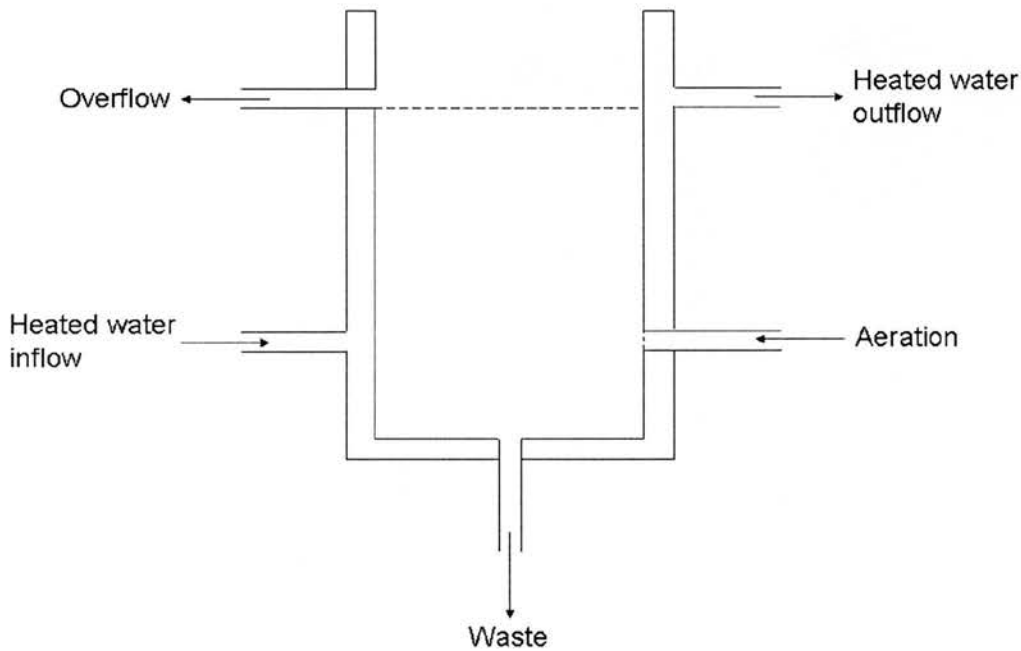
Materials and Methods**Chapter Two****2.1 Overview**

Female guinea pigs (weight range 250 – 400g) were killed by cervical dislocation in accordance with Schedule 1 of the UK Home Office regulations. The urinary bladder was removed en bloc, typically leaving a length of urethra 8 – 10mm long and ureters 6 – 8mm long, and then placed in Tyrodes solution. The solution was bubbled with a 5% CO₂ and 95% O₂ mixture (pH 7.4). The urethra was cannulated with a polyethylene cannula (external diameter 2mm) and secured distal to the bladder neck using a fine thread ligature. Residual urine was gently removed and replaced with Tyrodes solution. The bladder was then transferred to a heated organ bath (volume 30ml, temperature maintained at 33 - 36°C) containing constantly gassed Tyrodes solution. The cannula was connected via a fluid filled tube and 3 way connector to a pressure transducer (Becton Dickinson DTX Plus) and a 2.5ml syringe to enable variation of the intravesical volume. The transducer output was amplified, digitalised (sampling rate 20 Hz) and recorded using a Biopac MP100 data acquisition system, which gives the facility to capture a wide range of pressures from 0.02 to 80cmH₂O. The transducer was calibrated before each experiment. At the outset each bladder was filled with Tyrodes solution to give a baseline volume of 800 - 1200µl. These volumes were typically the amount of urine recovered from animals immediately after cervical dislocation. The time taken from killing the animal to beginning recording was typically 30 minutes. Pressure recording was begun immediately but the bladder was left to equilibrate for at least 20 min before any manipulations. Concentrated drug solutions were added directly to the bath to achieve the required final dilution. All drugs were added to the solution bathing the abluminal surface of the isolated whole bladder.

2.2 Materials

2.2.1 Organ bath and associated equipment:

Organ Bath: A jacketed, glass organ bath with an internal volume of 30ml. A schematic diagram is shown below. One port serves to act as an overflow, regulating the volume of Tyrodes solution to 30ml. Two ports are involved with the continual heating of the organ bath contents by the constant flow within the surrounding jacket of water heated to 33 - 36°C. One port allows the continual aeration of the solution within the organ bath with 5% CO₂ and 95% O₂. The final port allows drainage via gravity of the solution within the organ bath.



Water heater: electric thermostatically controlled water heater regulating temperature between 33 - 36°C. An inbuilt pump allows for continuous irrigation of heated water through the jacket surrounding the organ bath.

Cannula: A polyethylene cannula 2cm long with an external diameter of 2.0mm and internal luminal diameter of 1.5mm is introduced into the bladder via the urethra. A bevelled tip secures the cannula within the bladder once the urethra has been ligated.

Tubing: The length of tubing between the cannula and transducer was 50cm. To prevent dampening of transmitted pressure impulses stiff, inelastic polyethylene tubing was used. Internal diameter 1.5mm.

Calibration chamber: an open ended cylinder with a tapered tip allowing connection to a three way tap via a 50cm length of tubing. Measuring 1cm in diameter and 4cm tall. Held in position via a stand and clamp to allow calibration.

Three way tap: standard three way tap used in routine clinical practice. Allows an uninterrupted fluid connection to be made between either two or all of its connections. The tubing is fixed to the tap via a ‘luer’ lock.

2.2.2 Pressure recording and data acquisition system

Transducer (Becton Dickinson): Becton Dickinson DTX Plus transducer

Amplifier (Biopac, USA): General purpose single channel, differential amplifier module DA100C (Biopac, USA).

Specifications:	Gain:	50, 200, 1000, 5000
	Output range:	$\pm 10V$ (analogue)
	Freq response	

Low pass filter:	10Hz, 300Hz, 5000Hz.
High pass filter:	DC, 0.05Hz.
Input voltage (max):	$\pm 200\text{mV}$
Noise voltage:	$0.11\mu\text{V rms} - (0.05 - 10\text{Hz})$
Temperature drift:	$0.3\mu\text{V}/^\circ\text{C}$

Data acquisition unit (Biopac, USA): Data acquisition system MP100-CE (Biopac, USA) performing the same functions as a chart recorder allowing data to be digitalised before being recorded direct to and subsequently analysed upon a standard laptop computer.

Specifications:

Analogue inputs

Number of channels:	16
Input voltage range:	$\pm 10\text{V}$
A/D resolution:	16 bits
Accuracy (% of FSR):	± 0.003
Input impedance:	$1.0\text{M}\Omega$

Analogue outputs

Number of channels:	2
Output voltage range:	$\pm 10\text{V}$
D/A resolution:	12 bits
Accuracy (% of FSR):	± 0.02
Output drive current:	$\pm 5\text{mA}$
Output impedance:	$100\text{m}\Omega$

Time base

Min sample rate:	2 samples/ hour
Internal memory:	70K samples/ sec
PC memory/ disk:	11K samples/ sec
Internal buffer size:	16K samples
Serial interface rate:	800Kbits/ sec
Transmission type:	USB port type 1
USB interface:	Windows 98 (or above)

Laptop computer (Evesham, UK): a standard household laptop computer was used for data display, collection and analysis. 1.8GHz processor speed, utilising Windows XP® (Microsoft, USA) with a USB connection from the Biopac MP100-CE.

'AcqKnowledge' version 3.7.3 data interpretation programme (Biopac, USA): software package used to collect and interpret data on a standard laptop computer.

2.2.3 Consumables

Tyrodes solution: composition (mM): NaCl 120; KCl 4.5; CaCl₂ 2.5; MgCl₂ 1; NaHCO₃ 25; NaH₂PO₄ 1; sodium pyruvate 1, glucose 5. (pH 7.4 when constantly gassed with 5% CO₂ and 95% O₂).

2.2.4 Muscarinic agonists

Arecaidine but – 2 – ynyl ester tosylate (Tocris, UK): Potent muscarinic agonist, 4.6-fold selective for M₂ receptors in the atrium versus those in the ileum. Muscarinic activity M₂>M₃. (Barlow and Weston-Smith, 1985; Moser et al, 1989).

Carbamoylcholine chloride (Carbachol), (Sigma, UK): Non specific muscarinic agonist.

2.2.5 Muscarinic antagonists

[3H] – 4 – DAMP (Tocris, UK): Antagonist at the M₃ cholinergic receptor. [³H]-4-DAMP selectively labels M₃ and M₁ receptors. (Barlow and Shepherd, 1986; Michel et al, 1989).

AF-DX 116 (Tocris, UK): Selective M₂ muscarinic receptor antagonist. K_i values are 64, 417, 786, 211 and 5130nM for human recombinant M₂, M₁, M₃, M₄ and M₅ muscarinic receptors, respectively. (Hammer et al, 1986; Billard et al, 1995; Eglen et al, 1996; Mansfield et al, 2005).

2.2.6 Nicotinic antagonists

Hexamethonium Bromide (Sigma, UK): Nicotinic antagonist specific to receptors found on autonomic ganglia.

Pancuronium dibromide (Tocris, UK): Neuromuscular nicotinic receptor antagonist. (Fryer and Maclagan, 1987; Maestroni et al, 1994; Lowenick et al, 2001).

2.2.7 Purinoceptor agonist

2-Methylthioadenosine triphosphate tetrasodium salt (Tocris, UK): P2 purinoceptor agonist, a non-hydrolysable form of ATP. (Cusack et al, 1982; Burnstock et al, 1983; McMurray et al, 1998; Khakh et al, 2001).

2.2.8 Purinoceptor antagonists

PPADS tetrasodium salt (Tocris, UK): Non-selective P2 purinergic antagonist. Blocks recombinant P2X₁, P2X₂, P2X₃, P2X₅ (IC₅₀ = 1 – 2.6μM), native P2Y₂-like (IC₅₀ ~ 0.9μM), and recombinant P2Y₄ (IC₅₀ ~ 15μM) receptors. (Lambrecht et al, 1992; McLaren et al, 1994; Ziganshin et al, 1994).

MRS 2179 Tetrasodium salt (Tocris, UK): Competitive antagonist at P2Y₁ receptors (K_B = 100nM). Selective over P2X₁ (IC₅₀ = 1.15μM), P2X₃ (IC₅₀ = 12.9μM), P2X₂, P2X₄, P2Y₂, P2Y₄ and P2Y₆ receptors. (Boyer et al, 1998; Moro et al, 1998; Nandanan et al, 2000).

NF 279 (Tocris, UK): Selective P2X₁ antagonist (IC₅₀ = 19nM). Selective over P2X₂ (IC₅₀ = 0.76μM), P2X₃ (IC₅₀ = 1.62μM), P2X₄ (IC₅₀ > 300μM), and P2Y receptors. (Damer et al, 1998; Rettinger et al, 2000; Klapperstuck et al, 2000).

2.3 Methods

2.3.1 Preparation of the isolated guinea pig bladder

Experiments were performed using bladders isolated from female guinea pigs, weight range 250 – 400g. From delivery animals were stored in conditions complying with UK Home Office regulations, typically allowing a period of at least 48 hours acclimatisation before use.

2.3.2 Animal sacrifice

Animals were removed from their surroundings and killed immediately in accordance with Schedule 1 of the UK Home Office regulations. On all occasions cervical dislocation was used.

2.3.3 Dissection of the animal bladder

The time from cervical dislocation to the start of dissection was typically <4 minutes. A midline incision was made over the abdomen from the rib cage inferiorly to the pubis. Lateral extensions were made bilaterally at the inferior end of the incision to allow exposure of the abdominal contents. The small and large bowels were displaced superiorly exposing the genitourinary tract. Using heavy scissors a transverse cut was made across the animal, 1cm superior to the dome of the bladder, separating the upper abdomen/ thorax from the lower abdomen/ pelvis. For ease of dissection the pelvis was then pinned to a cork board and secured.

Once the pelvis was secured the bladder was dissected under magnification using light microscopy. (During this time the bladder was washed frequently with heated (33 - 36°C), gassed (5% CO₂ and 95% O₂) Tyrodes solution to prevent drying). Initially, the

pubic arch was transected both sides of the urethra and dissected free. Once the urethra was fully exposed it was mobilised off the underlying vagina. Keeping this plane, the bladder was subsequently dissected off the uterus. The ureters were identified bilaterally and dissected free from their enclosing fascia. The ureters, bladder and urethra were fully mobilised and were removed en bloc into a petrie dish containing heated, gassed Tyrodes solution. The urethra was cut to leave a length 8 – 10mm and the ureters to a length 6 – 8mm long. The ureters were not ligated as a length of 6 – 8mm was found to be sufficient to prevent leakage of the bladder contents. Overlying fat and fascia were excised from the bladder.

2.3.4 Cannulation of the animal bladder

The urethra was cannulated with a polyethylene cannula (external diameter 2mm) and secured 2-3mm distal to the bladder neck using a fine thread ligature. The cannula was placed such that the tip was positioned within the centre of the bladder. Residual urine was gently removed via the cannula and replaced with heated, gassed Tyrodes solution. Care was taken to remove air from the bladder.

2.3.5 Weighing of the animal bladders

The weight of the cannula, ligature, Tyrodes solution and the petrie dish were all known prior to the addition of the bladder. Residual urine removed from the bladder was kept separate and the Tyrodes solution used to replace this taken directly from the petrie dish. The cannulated bladder within the petrie dish was then weighed with the bladder weight being subsequently calculated. Mean bladder weight (\pm SEM) was $296 \pm 49\text{mg}$ ($n = 46$).

2.3.6 Transferring of the bladder to the organ bath

The bladder was placed within a heated organ bath (33 - 36°C) containing gassed (5% CO₂ and 95% O₂) Tyrodes solution. The cannula was attached to a fluid filled (Tyrodes solution) tube connecting to the transducer and data acquisition unit, and suspended upside down in the organ bath. (The urethra being superior and the bladder dome inferior). Again, care was taken to prevent air entering either the bladder or fluid filled tube during attachment. Typically, < 30minutes was taken from the time of cervical dislocation to transferring to the organ bath and starting pressure recording.

2.4 Preparation of pressure recording equipment

2.4.1 Setup of recording equipment

The equipment was setup prior to dissection of the animal bladder. A schematic diagram of the equipment is shown in Figure 12. The cannulated bladder was attached to a tube filled with Tyrodes solution connecting the bladder to a transducer and data acquisition system. Two '3 – way' taps were placed in the system, one to allow calibration of the transducer and the other to allow alteration in intravesical volume. Prior to attachment of the bladder the system was flushed with Tyrodes solution to remove air bubbles. The organ bath was also pre-filled with Tyrodes solution, gassed with 5% CO₂ and 95% O₂ (to give a pH of 7.4) and heated to 33 - 36°C.

2.4.2 Equilibration

Following attachment the bladder was gently emptied via the 2.5ml syringe attached to the system by way of a 3 way tap and then instilled with 1200µl of Tyrodes solution. The preparation was allowed to equilibrate for 20 minutes before any manipulations were made. Although, recordings were made as soon as calibration of the transducer had been performed these were not used for analysis.

2.4.3 Calibration of the transducer and data acquisition system

For calibration the fluid filled 'calibration' chamber was connected directly to the transducer via a '3 - way tap', with the connection to the bladder being closed. The output from the transducer and pressure recording by the data acquisition system (in cmH₂O) were calibrated against known heights of a standing column of fluid; in this case the height of the fluid level within the calibration chamber above the transducer. It

was assumed that the pressure from a standing column of water would be comparable to that of a standing column of Tyrodes solution.

2.4.4 Recording of intravesical pressure

Following calibration the transducer, by way of the '3 way tap', was reconnected to the bladder and the connection to the calibration chamber closed. Intravesical pressure measurements were continuously recorded in cmH₂O with a digital capture rate of 20Hz.

2.4.5 Assessment of minimal quality of experimental preparation

Following calibration of the equipment and equilibration of the organ system, the 'quality' of the isolated bladder was assessed. From the previous works of Gillespie and the authors own anecdotal experience it can be seen that the guinea pig bladder demonstrates typical responses following the addition of increasing doses of arecaidine, an example of a typical response to arecaidine is illustrated in Figure 17. Prior to the commencement of any experiments the response of the preparation to 30nM, 100nM and 300nM arecaidine was assessed. Those bladders that failed to respond in a typical fashion were discarded and not used for data collection. Approximately 15 bladders were discarded for this reason. 46 bladders were used for data collection throughout the course of these experiments.

2.5 Manipulation of the guinea pig bladder

2.5.1 Augmentation of Autonomous Activity:

In all experiments, except those assessing the effects of increasing doses of carbachol, arecaidine was used to augment autonomous activity. At steady state arecaidine was found to result in rises in intravesical pressure that were regular in both frequency and amplitude with little in the way of low amplitude irregular contractions when compared with carbachol. As such, arecaidine allowed for ease of interpretation of the effects of various compounds upon augmented activity as there was less inherent variability in the effects of the 'driving' muscarinic agonist. It is for this reason that the use of arecaidine has become widespread in these types of experiments, (Drake et al, 2003; Gillespie et al, 2003 through to 2006).

2.5.2 Addition of drugs

Concentrated drug solutions were added directly to the solute bathing the abluminal surface of the bladder to achieve the final desired concentration within the organ bath.

2.5.3 Removal of drugs ('washout')

The solution bathing the isolated bladder was drained via the 'waste port'. Fresh Tyrodes solution, pre-heated and gassed, was then immediately added to the organ bath. The organ bath was allowed to equilibrate for 180seconds prior to any further manipulations. At higher doses of drug concentrations further washouts were performed until the effects of the drug had been fully removed.

2.5.4 Alteration of intravesical volume

Intravesical volume was increased and decreased by a 2500 μ l syringe connected to the system via a 3 way tap. Prior to assessing the effects of muscarinic agonists/ antagonists on augmented activity intravesical volume was typically 1200 μ l. This volume remained fixed between these experiments. However, prior to experiments upon the 'volume response' intravesical volume was reduced to the minimum volume that would allow for a recordable variation in intravesical pressure, referred to as the 'baseline volume'. This was typically in the region of 200 – 400 μ l. Given the nature of measuring intravesical pressure via a fluid column, a minimum volume is required within the bladder to prevent collapsing of the bladder around the cannula tip and to allow transmission of pressure elevations.

2.5.5 Marking of time points

Each time the bladder was manipulated the time and nature of the manipulation was recorded on the data acquisition system.

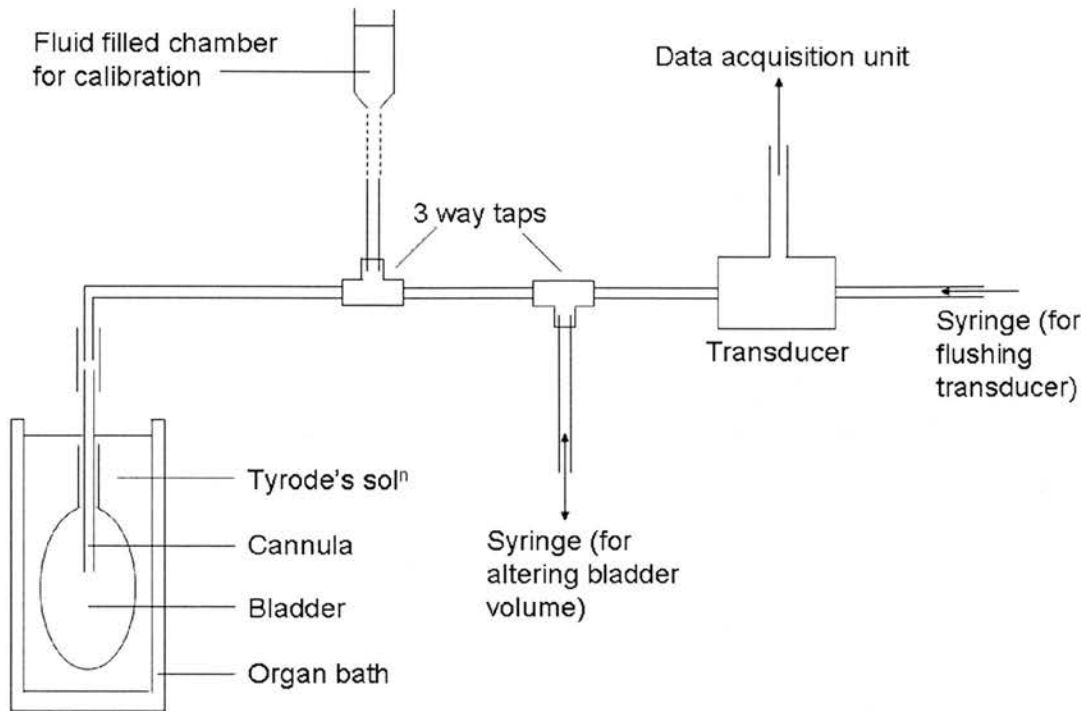


Figure 12: Schematic diagram demonstrating the setup used for recording intravesical pressure within the isolated guinea pig bladder. The bladder is suspended within the organ bath containing heated (33 - 36°C), gassed (5% CO₂ and 95% O₂) Tyrodes solution. The cannula within the bladder is connected to a transducer by fluid filled tubing 50cm in length. Two 3 - way taps are present. One connecting to a fluid filled chamber to allow calibration of the transducer, and the other to a 2.5ml syringe to allow manipulation of intravesical volume. The transducer is in turn connected to a data acquisition unit with inbuilt amplifier.

2.6 Data analysis

2.6.1 Number of data samples (n)

For all experimental analyses, only one set of data from each bladder was used. Typically this was taken from the first experiment of the day, immediately after equilibration, calibration and assessment of bladder quality. 'n' refers to the number of bladders used during the course of each experiment with only one set of data being taken from each bladder. (No more than one set of data was taken from a bladder).

2.6.2 Mean average, standard deviation (SD) and standard error of the mean (SEM)

The mean average and standard deviation were calculated using the basic statistics function of Excel (Microsoft ®). As calculation of the standard error of the mean (SEM) is not part of this software's application this was calculated manually within an Excel spreadsheet using the equation,

$$SEM = \frac{SD}{\sqrt{n}}$$

2.6.3 Paired Student's t-test

This test was performed to compare differences between groups of data obtained from the same isolated bladders where all data points were present. Excel (Microsoft ®) was used to calculate these. All results were 'two-tailed'.

2.6.4 t-test assuming unequal variances

This test was used to compare differences between data were either comparison of different bladders was made or data points were missing. Excel (Microsoft ®) was used to calculate these. All results were 'two-tailed'.

2.6.5 One way Analysis of variance (ANOVA)

In instances where multiple comparisons were performed across a range of different doses a one way analysis of variance was performed. To assess the significance of individual comparisons between doses Tukey's post hoc test was used to calculate significance and obtain a p-value. ANOVA and post hoc Tukey's tests were performed using the 'SPSS®' software package, SPSS inc.

2.6.6 Two way Analysis of Variance (ANOVA)

In instances where multiple comparisons were performed across a range of different doses and across two compounds a multi-factorial analysis, two way ANOVA, was performed. Again Tukey's post hoc test was used to calculate significance and obtain p-values. ANOVA and post hoc Tukey's tests were performed using the 'SPSS®' software package, SPSS inc.

RESULTS

Chapter Three

3.1 Introduction

During the course of these experiments two physiological responses inherent to the isolated guinea pig bladder were studied; 'Phasic Activity' (Drake et al, 2003a; Drake et al, 2003b) and the 'Volume Response' (Lagou et al, 2004). For each, data was acquired in the form of traces plotting intravesical pressure against time; a typical example of one of these traces is shown in Figure 13. Comparison between traces was possible qualitatively, allowing for gross differences and overall trends to be identified. However, in order for a quantitative assessment suitable for identification of subtle differences, in turn allowing formal statistical analysis, parameters specific to certain characteristics of the responses needed to be calculated, described below. Parameters common to both physiological responses are defined initially with parameters relating to a specific response being discussed at the beginning of the relevant results subsection. Mean values are expressed ± 1 standard error of the mean, significance is attributed to p values < 0.05 .

3.2 Interpretation of results

3.2.1 Definition and calculation of parameters common to spontaneous activity under iso-volumetric conditions and volume induced responses

(i) *frequency (transients/sec)*: frequency of phasic activity was determined by measuring the time interval between adjacent pressure rises, (b) – (a). The reciprocal of this interval represents the instantaneous transient frequency, at point (b), as the instantaneous frequency for a specific time point refers to the intertransient interval immediately preceding it. Therefore,

$$\text{freq at point (b)} = \frac{1}{(\text{time (b)} - \text{time (a)})} \quad (\text{Figure 13})$$

A plot showing the instantaneous frequency for the transients displayed in Figure 13 is illustrated, Figure 14.

(ii) *amplitude (cmH₂O)*: calculated as the pressure between the trough immediately preceding the transient contraction and the point of maximal contraction, ((c), Figure 13).

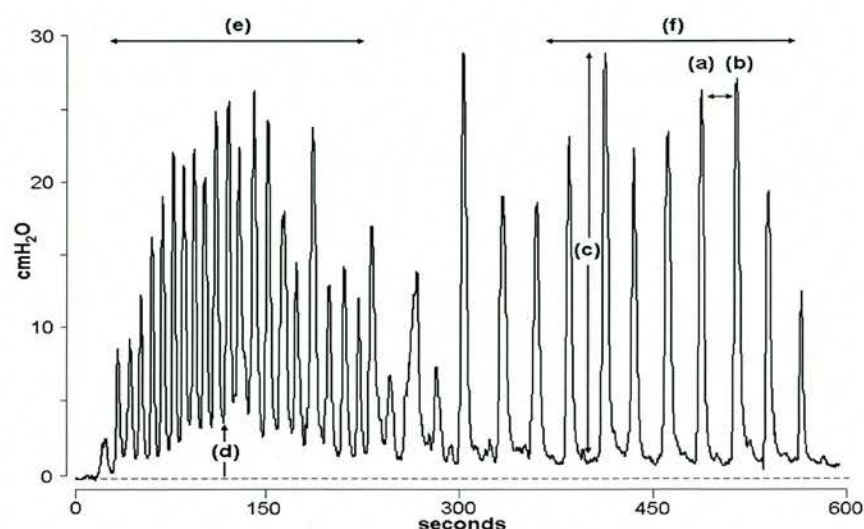


Figure 13: Typical trace plotting pressure (cmH₂O) against time (seconds); in this case showing the response following addition of 300nM Arecaidine to the solution bathing the isolated bladder. (b) – (a) highlights the time interval between adjacent pressure rises, (c) demonstrating the points used to calculate the amplitude of a transient contraction. (d) underlying pressure, (e) initial phase, (f) steady state.

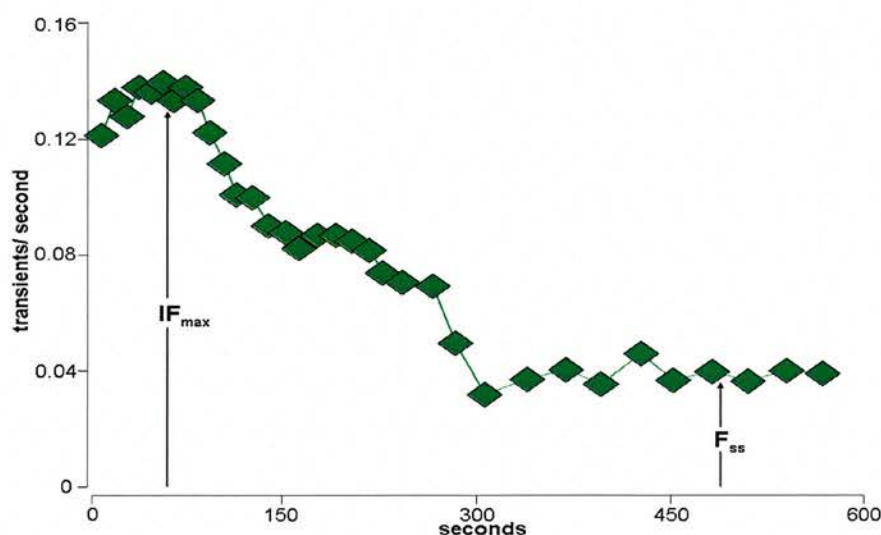


Figure 14: shows analysis of the record shown in Figure 13 illustrating the change in the frequency of transients during exposure to 300nM arecaidine. The data show the instantaneous frequency of the transients (defined as the reciprocal of the inter-spike interval) for each event in relation to the subsequent transient. Ordinate, frequency (transients/ second) and abscissa, time (seconds). Maximal initial frequency (IF_{max}) and frequency at steady state (F_{ss}) are shown.

3.2.2 Definition and calculation of parameters specific to spontaneous activity under iso-volumetric conditions

The addition of a muscarinic agonist to the isolated guinea pig bladder results in a series of complex changes in intravesical pressure, such as the effects of 300nM arecaidine demonstrated in Figure 13. Typically, an initial rise in basal pressure is seen with a superimposed increase in transient frequency. During prolonged exposure, the rise in basal pressure declines, the frequency of transients falls and the amplitude of transients increases. These two sets of events are consistent between the two agonists, arecaidine and carbachol, and as such parameters have been defined to help differentiate between these 'initial' and prolonged, 'steady state' phase,

- (i) *initial phase*: this is a qualitative term to represent the initial response of the preparation to the application of agonist. The transition between the initial phase and steady state varies between compounds and doses, making an exact demarcation between the two, and therefore a definition, difficult. However, ((e), Figure 13) highlights the area of increased frequency and rise in basal pressure typically referred to as the initial phase.
- (ii) *steady state*: refers to the time from which the frequency of transients and the basal pressure remains relatively constant, ((f), Figure 13).
- (iii) *maximum initial frequency (IF_{max})*: refers to the maximum instantaneous intertransient frequency seen following addition of agonist and is calculated from a frequency/ time plot for each experimental trace, (Figure 14). To calculate IF_{max} a line of best fit was added

manually to the freq/ time plot, taking into account overall trend and to exclude aberrant points.

(iv) *frequency at steady state (F_{ss})*: calculated in a manner similar to IFmax, F_{ss} refers to the relatively constant frequency seen following a prolonged exposure to an agonist. Again, due to some small variability in frequency a line of best fit was added manually by the researcher to calculate F_{ss} . An example of F_{ss} is shown in Figure 14.

(v) *maximal underlying pressure (P_{max})*: inspection of the responses, particularly at higher doses, reveals that the transient pressure rises do not fall back to basal pressure, i.e. the pressure prior to addition of agonist. Instead the transients appear to be superimposed upon an underlying rise in pressure. During the initial phase this includes a slow increase followed by a gradual decrease. Plots of pressure from baseline to the trough preceding each transient were constructed for each experiment, ((d), Figure 13). A line of best fit was drawn manually, by interpretation of the researcher, to include all the points of smallest amplitude, so to reduce the effects of aliasing, whereby pressure of the transients is not allowed to fall back to basal pressures due to the onset of another contraction. Maximal underlying pressure (P_{max}) refers to the maximal amplitude seen on the line of best fit. An example of an underlying pressure/ time plot for the trace seen in Figure 13 is shown below, (Figure 15).

(vi) *underlying pressure at steady state (P_{ss})*: as for P_{max} , P_{ss} is calculated from a line of best fit drawn on an underlying pressure/ time plot.

(vii) *Amplitude during the initial phase (A_{init})*: refers to the amplitude of transients ((c), Figure 13) during the initial phase. For calculation of the *mean of amplitudes* during the initial phase the amplitude of 2 transients either side of, and including, the transient at maximal frequency were used.

(viii) *Amplitude at steady state (A_{ss})*: refers to the amplitude of transients during the steady state.

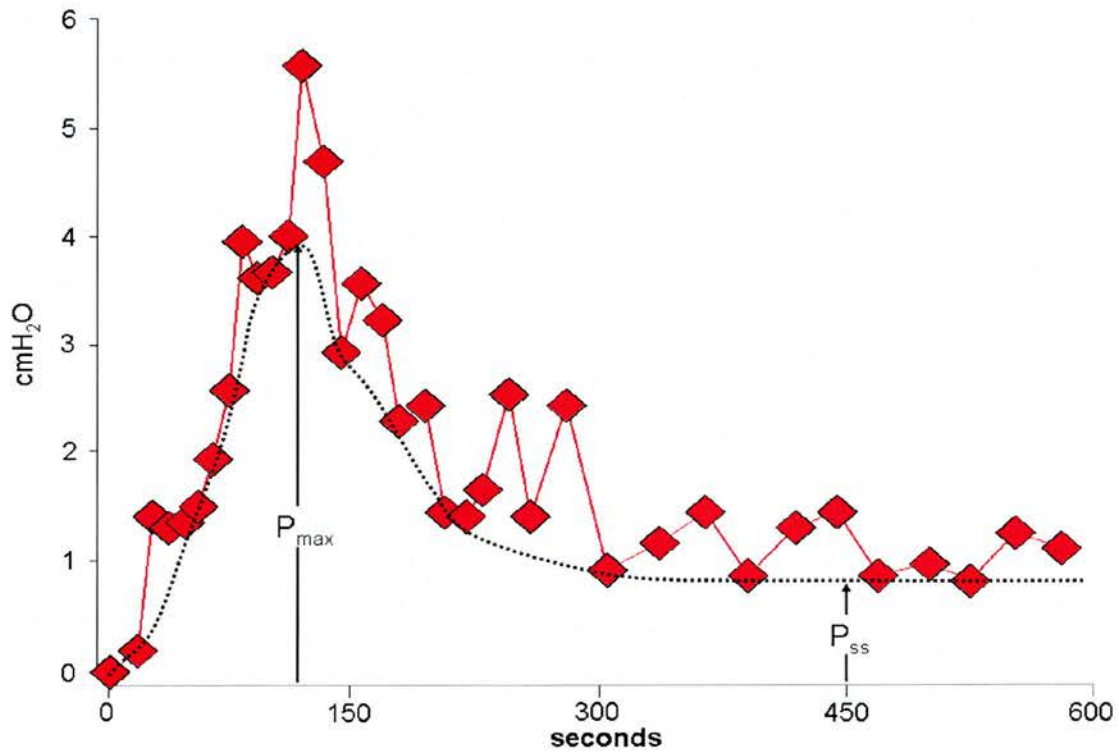


Figure 15: underlying pressure/ time plot for the trace seen in Figure 13. Ordinate, underlying pressure change from basal value (cmH₂O) and abscissa, time (seconds). Maximal underlying pressure (P_{max}) and underlying pressure at steady state (P_{ss}) are highlighted.

3.3 Effects of arecaidine and carbachol upon spontaneous activity

Addition of the muscarinic agonists arecaidine and carbachol resulted in the generation of augmented autonomous activity, consisting of regular transient contractions. Qualitatively, comparison of each drug individually revealed effects upon,

- frequency of transient contractions
- amplitude of transient contractions
- underlying pressure

For both agonists, amplitude and frequency of contractions increased at lower doses with underlying pressure increasing at doses $\geq 300\text{nM}$. Examples of typical traces illustrating the effects of increasing doses of carbachol and arecaidine are shown, figures 10 and 11 respectively. Clear distinctions between the 'initial' and 'steady state' phases are seen at doses $\geq 300\text{nM}$ carbachol and ≥ 100 arecaidine. To illustrate this phenomenon, an initial 'burst' of frequency settling down to a steady state upon prolonged exposure, traces of doses 30 – 300nM arecaidine shown in Figure 17 have been expanded, Figure 18 (a-c), with the instantaneous frequency for the transients charted for the three doses, Figure 18 (d).

Comparison of the traces for the two agonists reveals an overall trend, with frequency, amplitude and underlying pressure increasing with dose. However, differences exist mainly during the initial phase, between the agonists in their relative effects on these three variables. Figure 19 (a) and (b) illustrate typical instantaneous frequency plots derived from responses to different doses of carbachol (dose range 100-3000 nM) and arecaidine (dose range 30-3000 nM) respectively, taken from the same preparation. It

can be seen that with progressively higher concentrations of carbachol IF_{\max} increases. IF_{\max} also increases with arecaidine but to a lesser extent. Little difference in F_{ss} is seen between the two agonists. Figure 20 shows mean data accumulated from 6 different bladders, ($n=6$ bladder preparations), with standard error bars shown to 1 standard error of the mean. At 3000 nM carbachol, IF_{\max} was 0.36 ± 0.04 transients/sec, significantly greater than the value of 0.13 ± 0.01 transients/sec in 3000nM arecaidine ($p=0.02$). For 1000nM the means of IF_{\max} between carbachol and arecaidine were 0.21 ± 0.05 and 0.12 ± 0.007 respectively, ($p=0.048$). No significant difference was evident at doses ≤ 300 nM. In contrast, F_{ss} was not significantly different comparing carbachol with arecaidine at any concentration.

The effects of different concentrations of carbachol and arecaidine on the amplitude of the transients in the initial phase (A_{init}) and in the steady state (A_{ss}) are shown in Figure 20 c) and d). At low doses, <1000 nM, the amplitude of the transients generated by arecaidine, in both the initial phase (A_{init}) and steady state (A_{ss}) are significantly greater than those generated by carbachol. At 300nM mean A_{init} for arecaidine was 13.1 ± 2.2 cmH₂O, and for carbachol 3.4 ± 0.4 cmH₂O, ($p=0.002$). At 100nM mean A_{init} for arecaidine and carbachol were 8.63 ± 1.4 and 1.4 ± 0.46 cmH₂O respectively, ($p=0.001$). A similar pattern was also seen with mean amplitudes at steady state (A_{ss}). At 300nM mean A_{ss} for arecaidine was 20.8 ± 2.7 cmH₂O, and for carbachol 7.3 ± 1.1 cmH₂O, ($p=0.001$). At 100nM A_{ss} for arecaidine and carbachol were 12.3 ± 2.2 and 1.6 ± 0.42 cmH₂O respectively, ($p=0.002$). No significant difference in amplitudes of transient contractions during either the initial or steady state phases was seen between arecaidine and carbachol at doses ≥ 1000 nM.

Figure 20 e) and f) illustrate the effects of carbachol and arecaidine in the apparent underlying contraction in the initial phase (P_{init}) and steady state (P_{ss}). Apart from at a dose of 300nM, for both compounds, there appears to be no significant difference in the amplitude of the rise in underlying pressure generated by carbachol or arecaidine.

An interesting observation to note is that at higher doses, $\geq 1000\text{nM}$, both agonists show a dip in frequency at around 350 seconds to levels below those generated by lower doses of agonist. This reduction slowly increases back to basal levels by 550 seconds; the significance of this 'undershooting' phenomenon is uncertain but its presence is best highlighted by the addition of 1000nM arecaidine illustrated in Figure 19 b).

The addition of muscarinic agonist to the isolated bladder gives a complex response consisting of two distinct phases, suggesting the presence of two distinct but interconnected cholinergically processes involved in the regulation of spontaneous bladder activity. The frequency of the initial phase is significantly increased at doses of 1000nM carbachol and higher with no significant difference seen with any dose of arecaidine. This would suggest that initial frequency is mediated by an M_3 response. The fact that carbachol has little effect upon the frequency at steady state could signify either that there is little to no M_3 mediation of the frequency at steady state or that there is little cholinergic mediation of steady state frequency since arecaidine also has no significant effect. However, regulation of the amplitude of spontaneous contraction seems to be more M_2 mediated since there is a significant increase in amplitudes both initially and at steady state, at doses of 300nM or less. The insignificant differences at

doses of 1000nM and above may be result of the non-specific M₂ effects of carbachol at higher doses.

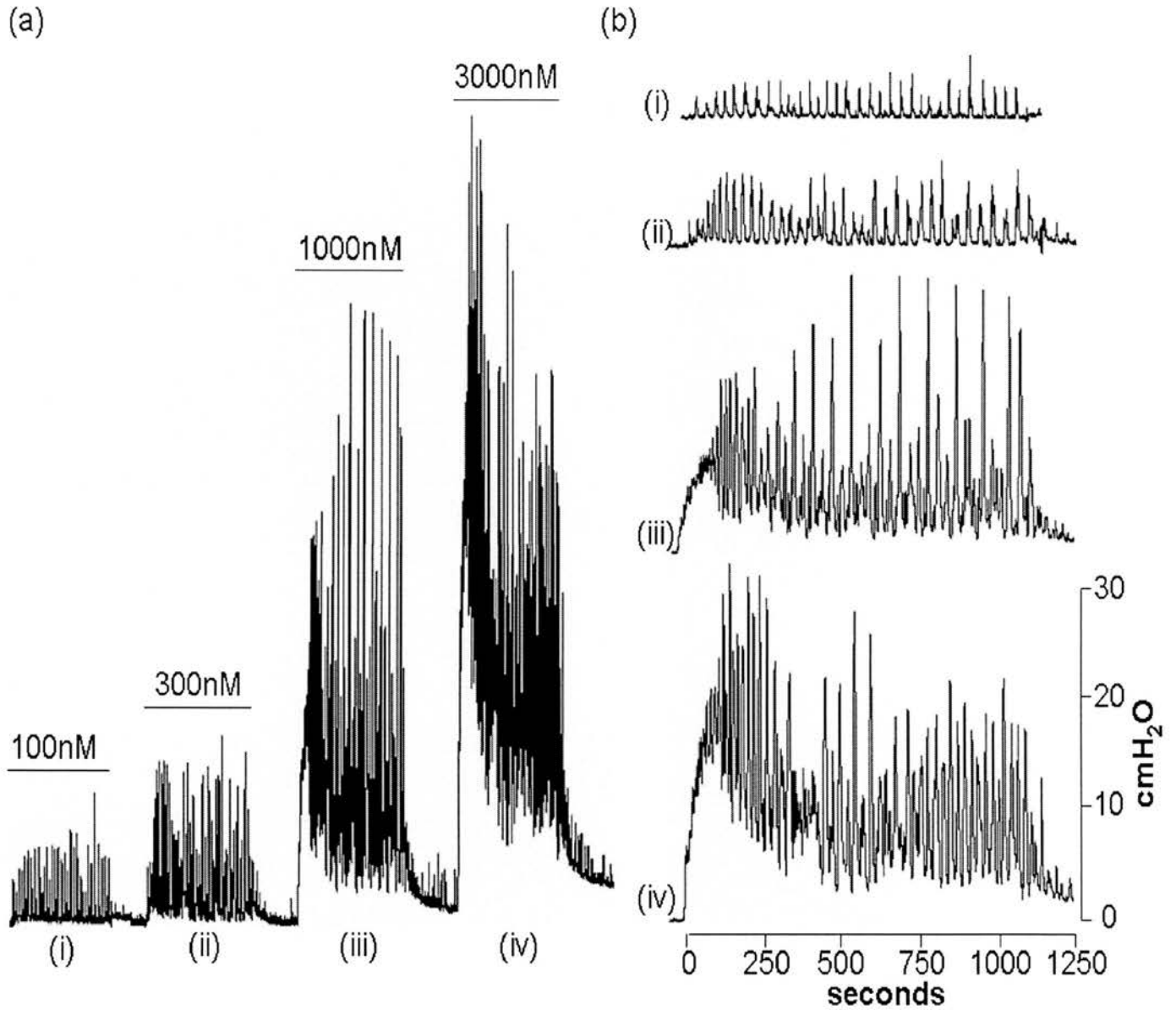


Figure 16: Dose dependent actions of carbachol on an isolated guinea pig bladder. a) shows the effects resulting from different concentrations of carbachol added to the bathing solution. Carbachol was added at the concentration shown during the time indicated by the horizontal bar. Between drug applications the bladder was washed in Tyrode's solution. (b) shows the records illustrated in (a) for each concentration of carbachol on an expanded time scale. Different components to the responses can be seen. At the low doses, 100 nM and 300 nM, the most obvious action is the generation of small transient rises in intra-vesicle pressure. At higher concentrations, 1000 nM and 3000 nM there is a clear initial burst of high frequency phasic activity which is superimposed on a rise in basal pressure. After this initial burst of high frequency activity the frequency of the transients declines to a constant level. (Note the complex and varied amplitude of the phasic activity during exposure to the highest concentrations of carbachol). In all panels the ordinates show pressure in cmH₂O and abscissae time in seconds.

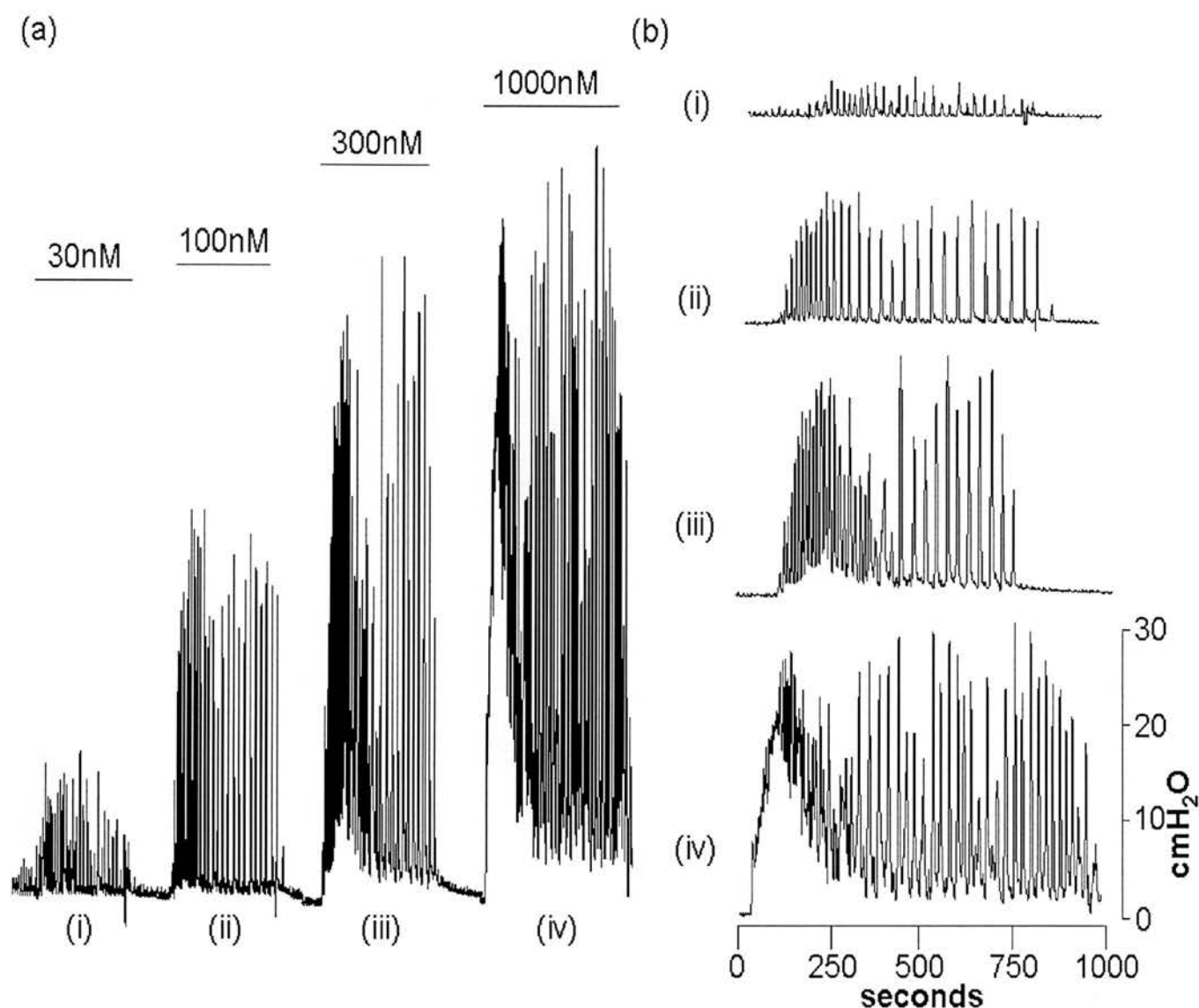


Figure 17: shows similar responses for different concentrations of arecaidine. (a) shows the responses resulting from application of 4 different concentrations. (b) shows the same responses but on an expanded time scale. In all panels the ordinates show pressure in cmH₂O and abscissae time in seconds.

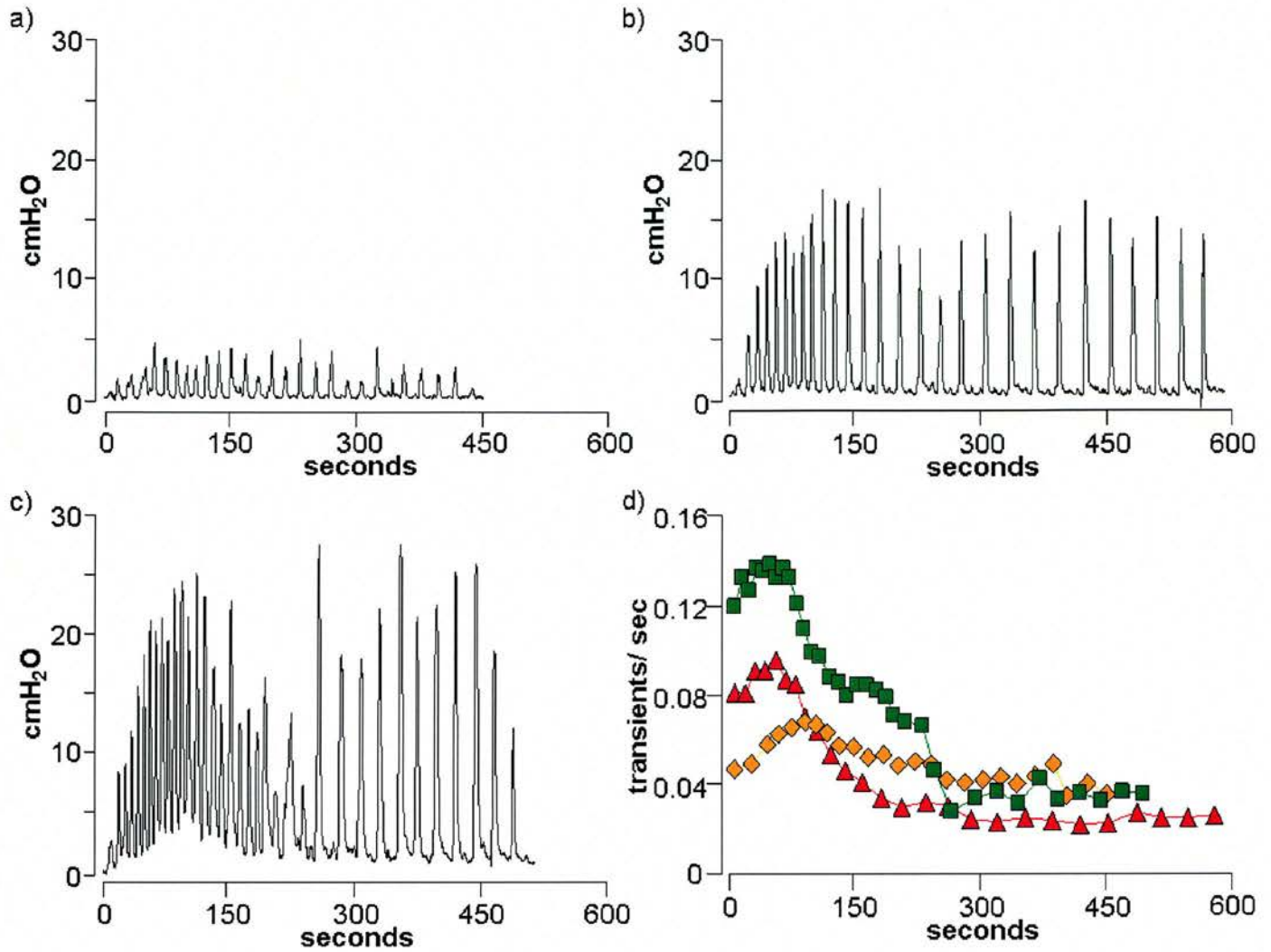


Figure 18: A detailed analysis of the response of the whole bladder to arecaidine. a) – c) show the records of the responses generated by 30, 100nM and 300nM arecaidine respectively. d) shows analysis of these records illustrating change in the instantaneous frequency of the transients during exposure to arecaidine, (300nM (■), 100nM (▲), and 30nM (◆)). The data show the instantaneous frequency of the transients (defined as the reciprocal of the inter-spike interval) for each event in relation to the previous transient. Ordinates in a) – c) pressure (cmH₂O) and d) frequency (transients/ second) and abscissae for all panels, time (seconds).

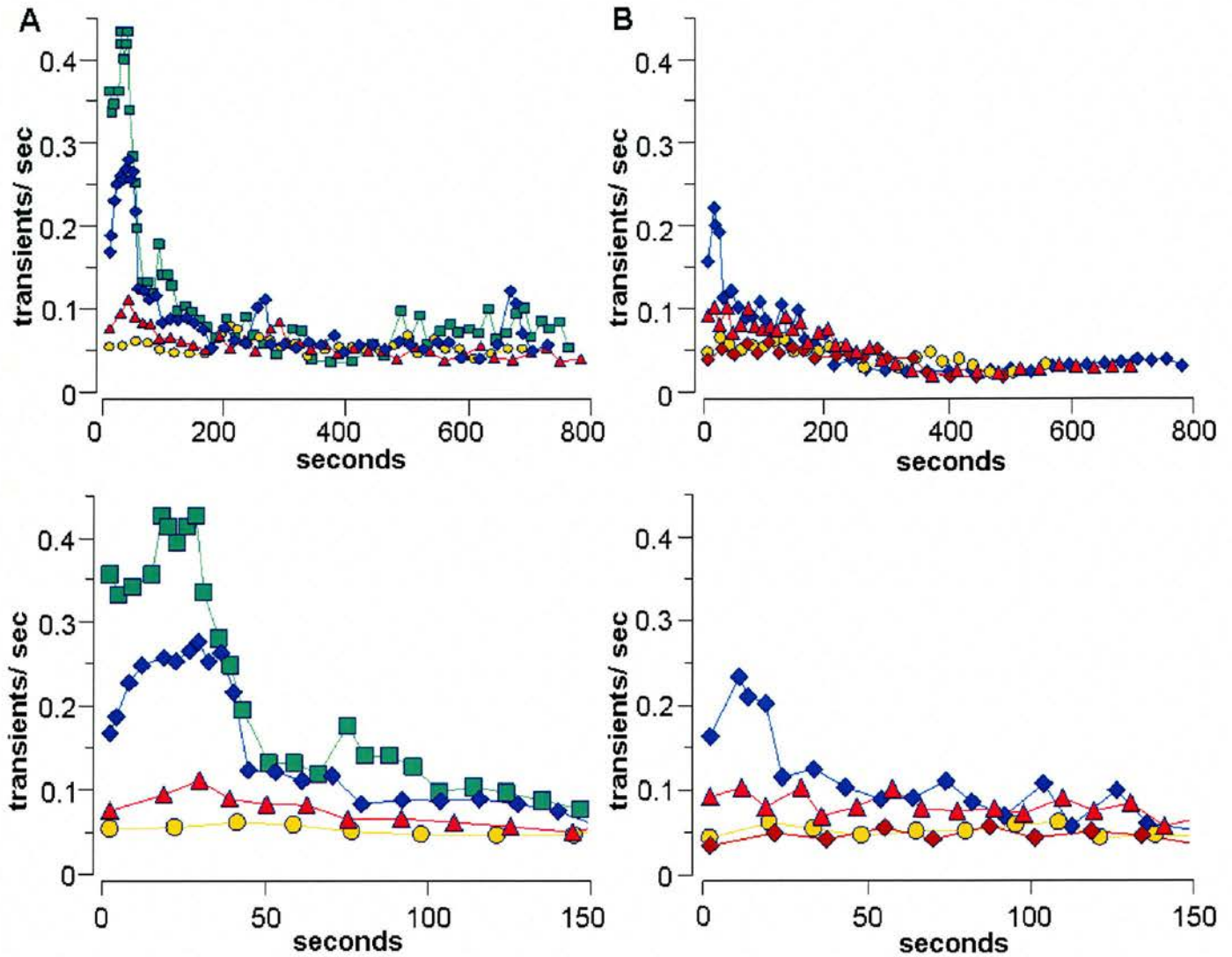


Figure 19: The effects of different concentrations of carbachol and arecaidine on the frequency of augmented spontaneous activity. A shows, from a single experiment, data derived from exposure to 4 different concentrations of carbachol. The upper panel show the data during the entire exposure to carbachol lasting 600 seconds. The initial 150 seconds of these responses are shown in the panel below. B shows data from the same bladder exposed to different concentrations of arecaidine. The upper panel show the data during an exposure to arecaidine lasting 600 seconds. The initial 150 seconds of these responses are shown in the panel below. Ordinates, frequency (transients/ second) and abscissae, time (seconds). For both agonists, 3000nM (■), 1000nM (blue ♦), 300nM (▲), 100nM (●), and 30nM (brown ♦).

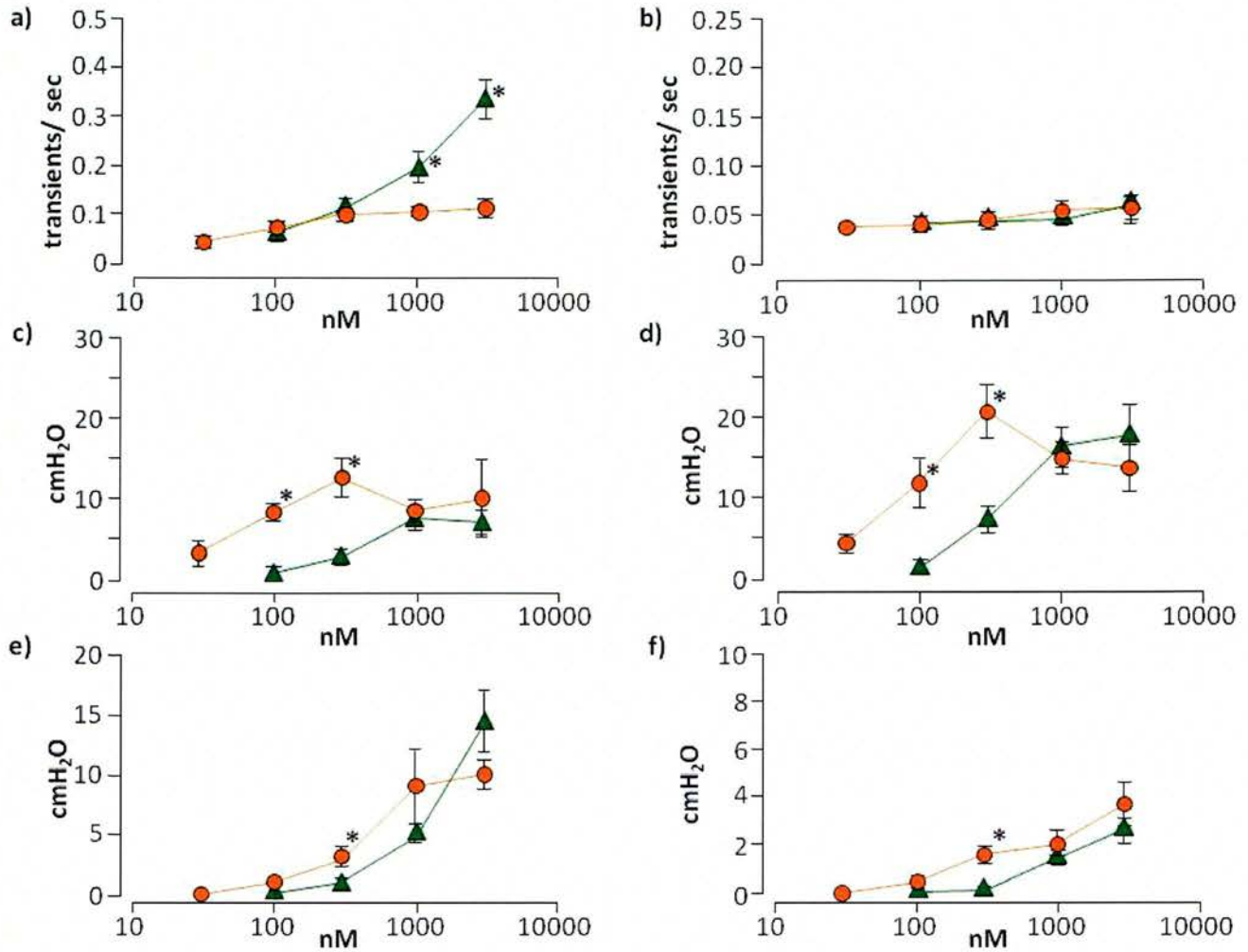


Figure 20: Data accumulated from 6 different bladders illustrating the effects of carbachol and arecaidine on the frequency and amplitude of the transients and the initial rise in basal pressure. a) and b) show the effects of carbachol (Δ) and arecaidine (O) on the initial and steady state frequencies respectively. c) and d) show the effects of the agonists on the amplitude of the transient rises in intra-vesical pressure in the initial phase and steady state respectively. e) and f) show data derived for the underlying rises in basal pressure in the initial phase and in the steady state respectively. Data are shown as mean values \pm 1 SEM (n=6). Ordinates, a) – b) frequency (transients/ second), c) – f) pressure (cmH₂O) and abscissae, \log_{10} agonist concentration (nM). A significant difference between the data for carbachol and arecaidine is indicated (* denotes a statistically significant difference between doses; $p < 0.05$, Two way ANOVA and post hoc Tukey's).

3.4 Effects of 4-DAMP and AF-DX upon spontaneous activity

Using the response of the isolated guinea pig bladder to 1000nM arecaidine, as described previously, as a control the effects of each of the two muscarinic antagonists, 4-DAMP ($M_3 > M_2$) and AF-DX ($M_2 > M_3$), were compared. An example of typical responses of 1000nM arecaidine in the presence of increasing doses of 4-DAMP is shown in Figure 21 A. This shows the original records taken from one bladder, Figure 21 A. A control response to a 5 minute exposure of 1000nM arecaidine was performed (control) with further responses performed in the same concentration of arecaidine but in the presence gradually increasing doses of 4-DAMP. A washout of pre-heated/ carboxygenated Tyrode's solution was performed between each dose increase. Figure 21 B shows each of the responses shown in A on an expanded time scale. Figure 21 C shows the instantaneous frequency plots for each dose over the first 250 seconds of the response, illustrating the effects of increasing doses of 4-DAMP during the 'initial' phase.

From cursory examination of the traces it is clear that 4-DAMP, at low concentration, had a marked effect on the responses generated by 1000nM arecaidine. Close examination of these responses demonstrate that the frequency of the initial burst of activity (IF_{max}) to be reduced by increasing concentrations of 4-DAMP as well as the amplitude, both initially and at steady state. The underlying pressure also seems to fall with increasing doses.

Figure 22 shows an analysis of the experiments illustrated in Figure 21, performed upon six bladders, ($n=6$). The mean values along with error bars representing 1 standard error of the mean are shown. Figures 16 a) and b) show mean values for

maximum initial frequency (IF_{max}) and frequency at steady state (F_{ss}) respectively. At low concentrations, 0.3nM, there is a significant reduction in IF_{max} . As the concentration of 4-DAMP is increased the reduction in IF_{max} becomes more pronounced, appearing to reach a minimum value around 10nM 4-DAMP of 0.07 transients per second. In contrast, the effect of 4-DAMP on F_{ss} is less marked. It is noted that the lowest value of IF_{max} in 10nM 4-DAMP (0.069 transients/ second) approaches the values of F_{ss} . Thus, it is therefore possible that a component responsible for the initial fast component/ burst of activity has almost been removed leaving the slow component seen during the steady state relatively unaffected by 4-DAMP. If this is the case then concentrations of 4-DAMP as low as 0.03nM are sufficient to reduce the initial frequency by 50%. (Suggesting the mechanisms generating the initial transients are particularly sensitive to inhibition by M_3 specific antagonists). Values for the mean IF_{max} in the presence of increasing doses of 4-DAMP are shown in table 4. No significant effect was seen upon frequency at steady state (F_{ss}) at doses up to 10nM.

Dose (nM)	Mean IF_{max} (transients/ sec), \pm 1 SEM	P value
0	0.137 ± 0.008	-
0.1	0.128 ± 0.004	0.48
0.3	$0.112 \pm 0.006^*$	0.042
1	$0.096 \pm 0.004^*$	0.02
3	$0.083 \pm 0.01^*$	0.01
10	$0.069 \pm 0.007^*$	0.007

Table 4: showing the mean effects of increasing doses of 4-DAMP (nM) upon the maximum initial frequency (IF_{max}), (transients/ second), calculated from six bladders, (n=6). A statistically significant reduction compared to control ($p<0.05$) is illustrated by an asterix (*) with the respective p value shown in the third column (one way ANOVA and post hoc Tukey's).

Mean values for maximal underlying pressure (P_{max}) and underlying pressure at steady state (P_{ss}) are shown in figures 16 c) and d) respectively. A significant reduction in P_{max}

is seen at doses $> 0.3\text{nM}$ 4-DAMP with the amplitude approaching 0 by 10nM . Mean values (± 1 standard error of the mean) for P_{max} are shown in table 5. However, the only significant reduction in P_{ss} was seen at 10nM 4-DAMP, where again it approached 0. Mean control P_{ss} was $0.87 \text{ cmH}_2\text{O} \pm 0.09$, with a P_{ss} at 3nM 4-DAMP $0.41 \text{ cmH}_2\text{O} \pm 0.2$, ($p=0.12$). However, mean P_{ss} in the presence of 10nM 4-DAMP was $0.025 \text{ cmH}_2\text{O} \pm 0.02$, a significant difference between control, ($p=0.006$).

Dose (nM)	Mean P_{max} (cmH_2O), ± 1 SEM	P value
0	4.45 ± 0.52	-
0.1	4.3 ± 0.7	0.36
0.3	2.8 ± 0.28	0.11
1	2.24 ± 0.51	0.08
3	$1.81 \pm 0.78^*$	0.042
10	$0.19 \pm 0.043^*$	0.007

Table 5: showing the mean effects of increasing doses of 4-DAMP (nM) upon the maximum underlying pressure (P_{max}), (cmH_2O), calculated from six bladders, ($n=6$). A statistically significant reduction compared to control ($p<0.05$) is illustrated by an asterisk (*) with the respective p value shown in the third column (one way ANOVA and post hoc Tukey's).

Figures 16 e) and f) show values for the mean amplitude of transient contractions during the initial phase, mean A_{init} , and at steady state, mean A_{ss} . A significant reduction in mean A_{init} was seen at 10nM only. Mean control A_{init} was $5.6 \text{ cmH}_2\text{O} \pm 0.67$ and mean A_{init} in the presence of 10nM 4-DAMP was $1.5 \text{ cmH}_2\text{O} \pm 0.37$, ($p=0.008$). For mean A_{ss} a significant reduction was seen at doses of 4-DAMP $\geq 3\text{nM}$. Mean A_{ss} control $14.8 \text{ cmH}_2\text{O} \pm 1.5$, 3nM 4-DAMP 9.1 ± 1.72 ($p=0.044$), and 10nM 4-DAMP 2.8 ± 0.51 ($p=0.0016$).

The effects of 4-DAMP upon spontaneous activity stimulated by 1000nM arecaidine reveal that the frequency generated in the initial phase is strongly M_3 mediated since 4-

DAMP significantly reduces initial frequency even at small doses, 0.3nM. However, it has little effect upon the frequency at steady state, again suggesting little M_3 regulation of the frequency of spontaneous activity once the initial phase has passed. The effects of M_3 mediation in the generation of underlying activity may be a direct result of M_3 mediated contraction however, in the initial phase it is likely to be related to a degree of aliasing whereby transients are unable to return to baseline values since the frequency of subsequent contractions are too high. With a reduction in frequency a reduction in basal pressure is therefore automatically seen. In addition, the reduction in underlying contraction at 10nM 4-DAMP could represent a non specific M_2 response.

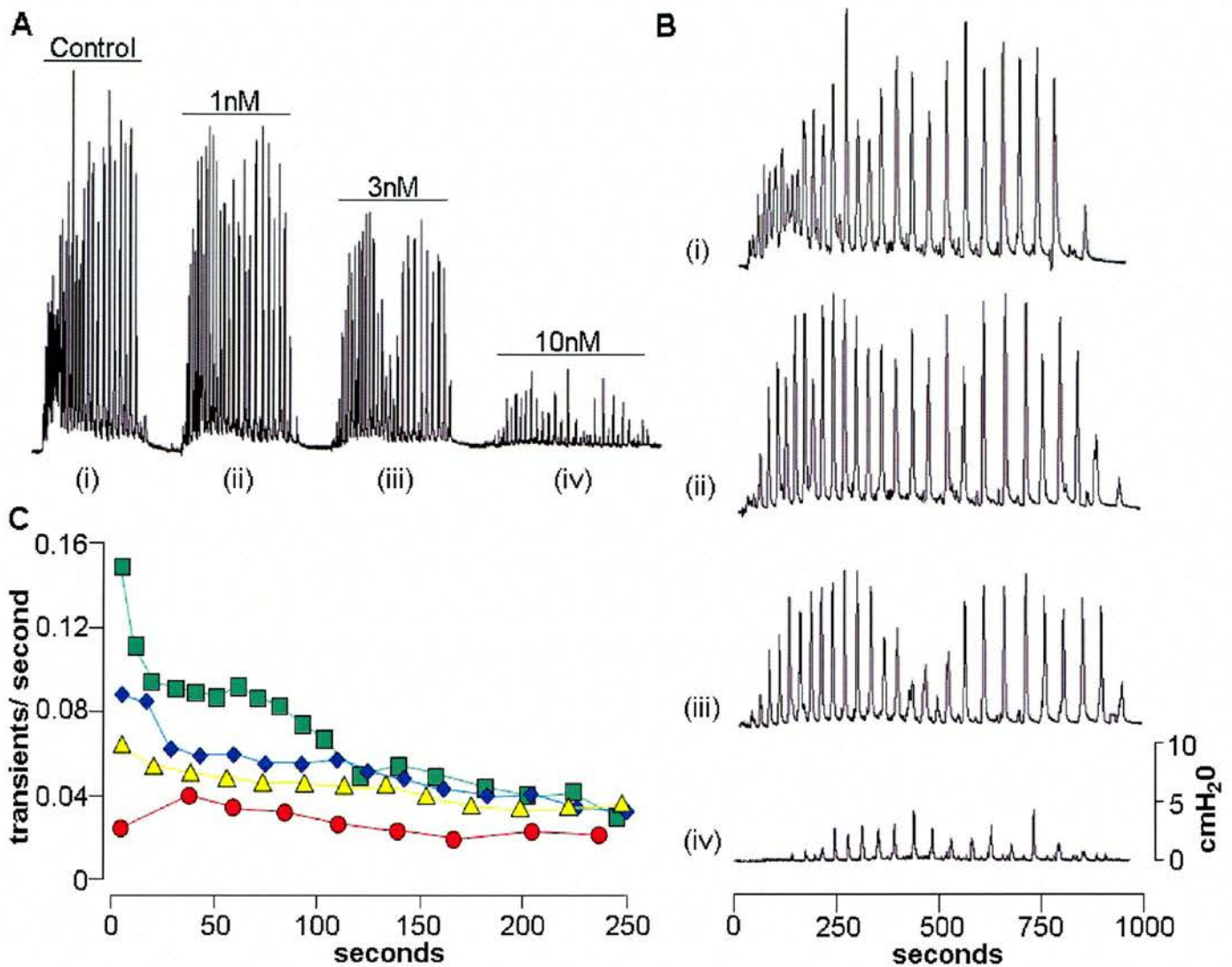


Figure 21: Dose dependent actions of the M_3 specific antagonist 4-DAMP on an isolated guinea pig bladder in the presence of 1000nM arecaidine. A shows the effects resulting from adding 1000nM arecaidine to the bathing solution in the presence of different concentrations of 4-DAMP. Between arecaidine applications the bladder was washed in Tyrode's solution containing the next incrementally increased dose of 4-DAMP. B shows the records illustrated in A for each concentration of 4-DAMP on an expanded time scale. C shows the instantaneous frequency plot for the first 250 seconds following addition of arecaidine in the presence of doses 0 - 10nM 4-DAMP, (control (■), 1 nM (◆), 3 nM (▲) and 10 nM (●)). These illustrate the different components of the responses. At low doses, 1nM, the most obvious action is the reduction in frequency during the initial phase. At higher concentrations, 3nM and 10nM, there is a reduction in amplitude and underlying pressure in both the initial phase and at steady state. In panels A and B the ordinates show pressure in cm H₂O and abscissae time in seconds. Panel C ordinate, frequency (transients/ second) and abscissa, time (seconds).

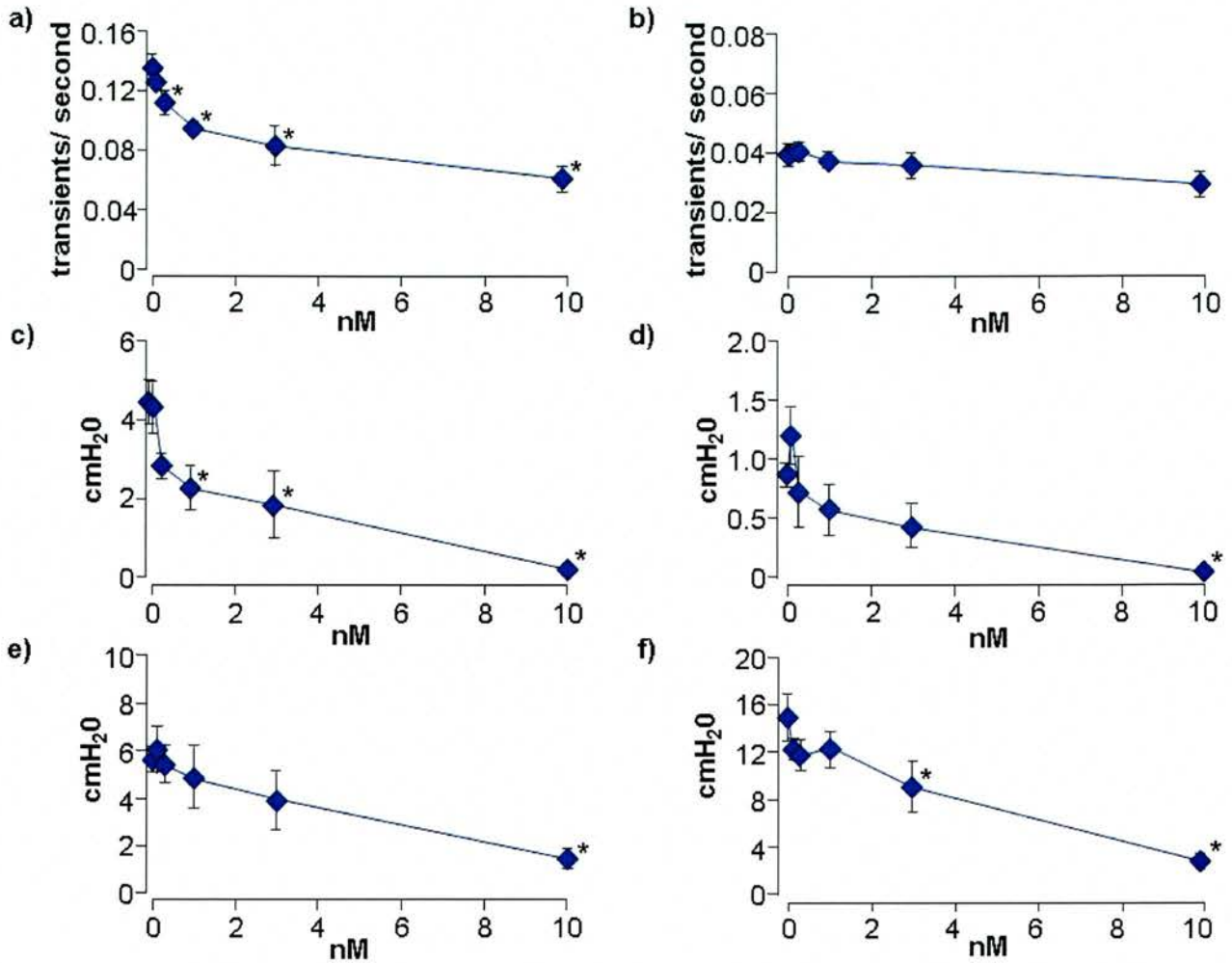


Figure 22: Data accumulated from 6 bladders illustrating the effects of different concentrations of the M₃ antagonist 4-DAMP on arecaidine induced responses. a) and b) illustrate the effects of 4-DAMP on the maximum initial frequency (IF_{max}) and frequency at steady state (F_{ss}) respectively. c) and d) illustrate the effects of 4-DAMP on the initial (P_{init}) and steady state (P_{ss}) rises in basal pressure respectively. e) and f) show the effects on the mean amplitudes of the initial (A_{init}) and steady state (A_{ss}) transients respectively. Data are shown as mean values ± 1 SEM (n=6). Ordinates, a) and b) frequency (transients/sec), c) – f) pressure (cmH₂O) and abscissae, antagonist concentration (nM). A significant difference between the data points compared to control is indicated (*), (p<0.05, one way ANOVA and post hoc Tukey's).

In examining the effects of the relatively M_2 specific antagonist AF-DX a different pattern of responses is seen. Figure 23 shows a typical response to 1000nM arecaidine in the presence of increasing doses of AF-DX; following the same experimental protocol as described for 4-DAMP. It illustrates little effect of AF-DX upon the instantaneous frequency of transients during the initial phase. Though the amplitude of transient contractions and the underlying pressure seem to be reduced at higher doses. Figure 23 A illustrates traces taken from a typical experiment. Figure 23 B shows each of the responses on an expanded time scale. Figure 23 C shows the instantaneous frequency plots for each dose for 600 seconds following addition of 1000nM arecaidine, highlighting relatively little effect of increasing doses of AF-DX on the 'initial' phase.

Figure 24 shows an analysis of the experiments illustrated in Figure 23. The effects of AF-DX were studied upon six bladders, ($n=6$), and mean values are expressed along with error bars representing 1 standard error of the mean. Figures 18 a) and b) show mean values for maximum initial frequency (IF_{max}) and frequency at steady state (F_{ss}) respectively. Neither IF_{max} nor F_{ss} were appreciably affected by AF-DX; IF_{max} was only reduced significantly at 1000nM and no significant reduction was seen upon F_{ss} .

Figures 18 c) and d) show the effects of AF-DX upon maximal underlying pressure (P_{max}) and underlying pressure at steady state (P_{ss}) respectively. Mean P_{max} was significantly reduced at doses ≥ 30 nM. No significant reduction was seen upon P_{ss} . Table 6 shows mean P_{max} (± 1 SEM) for increasing doses of AF-DX.

Dose (nM)	Mean P_{\max} (cmH ₂ O), ± 1 SEM	P value
0	8.18 \pm 0.43	-
30	5.39 \pm 0.14*	0.048
100	3.63 \pm 0.15*	0.032
300	1.91 \pm 0.08*	0.037
1000	0.98 \pm 0.20*	0.021

Table 6: showing the mean effects of increasing doses of AF-DX (nM) upon the maximum underlying pressure (P_{init}), (cmH₂O), calculated from six bladders, (n=6). A statistically significant reduction compared to control ($p < 0.05$) is illustrated by an asterix (*) with the respective p value shown in the third column (one way ANOVA and post hoc Tukey's).

Mean amplitudes of transient contractions during the initial phase, mean A_{init} , and at steady state, mean A_{ss} are shown in figures 18 e) and f). For mean A_{init} significant reductions were seen at doses of AF-DX $\geq 1000\text{nM}$, table 7. Significant reduction in mean A_{ss} was also only seen at 1000nM AF-DX.

Dose (nM)	Mean A_{init} (cmH ₂ O), ± 1 SEM	P value
0	9 \pm 0.23	-
30	6 \pm 1.10	0.47
100	7.4 \pm 0.92	0.56
300	2.7 \pm 0.23	0.054
1000	2.35 \pm 0.03*	0.032

Table 7: showing the mean effects of increasing doses of AF-DX (nM) upon the mean amplitude of contraction during the initial phase (A_{init}), (cmH₂O), calculated from six bladders, (n=6). A statistically significant reduction compared to control ($p < 0.05$) is illustrated by an asterix (*) with the respective p value shown in the third column (one way ANOVA and post hoc Tukey's).

A direct comparison was made between the data obtained with 4-DAMP and AF-DX.

Figure 25 combines the data from figures 16 and 18. It is clear that 4-DAMP is dose for dose, more effective than AF-DX in affecting all parameters investigated. However, it

is noteworthy that the frequency of the transients in the steady state are only marginally affected.

AF-DX was not seen to have any significant effect upon frequency during either the initial phase or at steady state; except at 1000nM during the initial phase. This would suggest that the mechanisms involved in the generation of frequency have little in the way of M_2 mediation. The significant reduction seen in the amplitude of the spontaneous contractions seen during the initial phase suggests a degree of M_2 mediation of the mechanisms involved in the generation of contractions as opposed to an aliasing effect since AF-DX has little effect upon frequency.

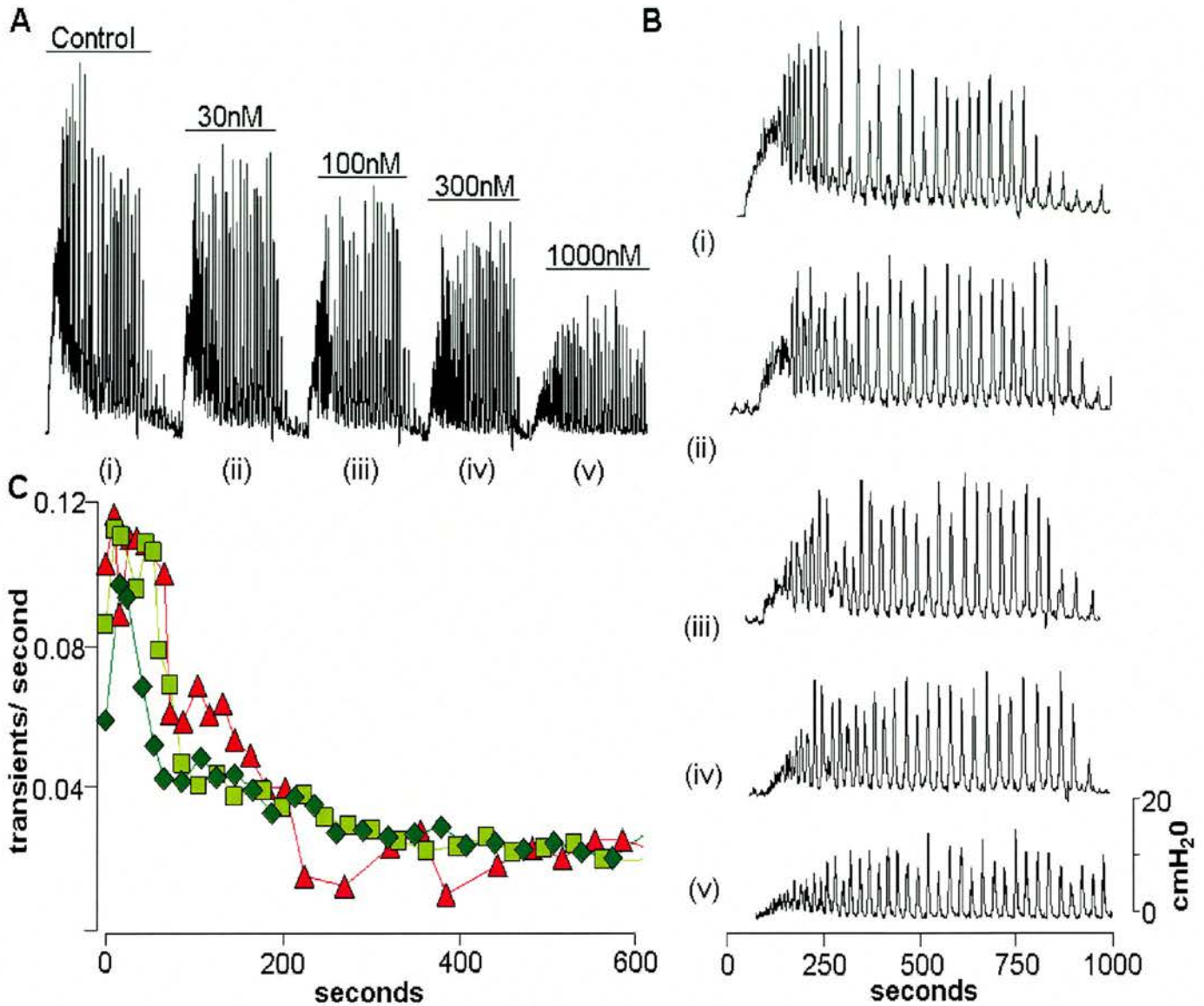


Figure 23: Dose dependent actions of the M₂ specific antagonist AF-DX on an isolated guinea pig bladder in the presence of 1000nM arecaidine. A shows the effects resulting from adding 1000nM arecaidine to the bathing solution in the presence of different concentrations of AF-DX. Between arecaidine applications the bladder was washed in Tyrode's solution containing the next incrementally increased dose of AF-DX. B shows the records illustrated in A for each concentration of AF-DX on an expanded time scale. C shows the instantaneous frequency plot for the first 600 seconds following addition of arecaidine in the presence of doses 0 - 1000nM AF-DX, (control (▲), 300 nM (■) and 1000 nM (◆)). In panels A and B the ordinates show pressure in cm H₂O and abscissae time in seconds. Panel C ordinate, frequency (transients/ second) and abscissa, time (seconds).

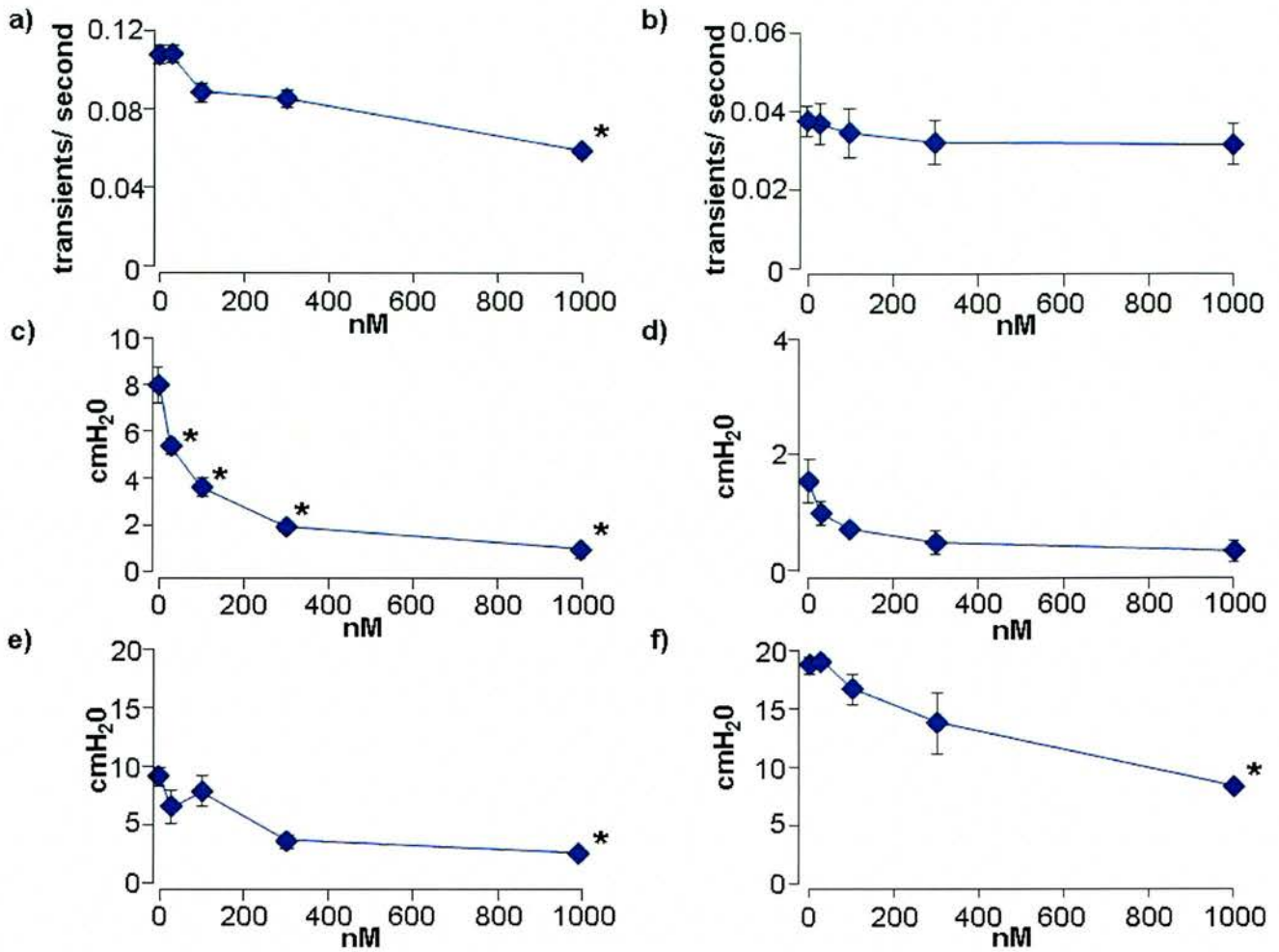


Figure 24: Data accumulated from 6 bladders illustrating the effects of different concentrations of the M₂ antagonist AF-DX on arecaidine induced responses. a) and b) illustrate the effects of AF-DX on the maximum initial frequency (IF_{max}) and frequency at steady state (F_{ss}) respectively. c) and d) illustrate the effects of AF-DX on the initial (P_{init}) and steady state (P_{ss}) rises in basal pressure respectively. e) and f) show the effects on the mean amplitudes of the initial (A_{init}) and steady state (A_{ss}) transients respectively. Data are shown as mean values \pm 1 SEM (n=6). Ordinates, a) and b) frequency (transients/sec), c) – f) pressure (cmH₂O) and abscissae, antagonist concentration (nM). A significant difference between the data points compared to control is indicated (*), (p<0.05, one way ANOVA and post hoc Tukey's).

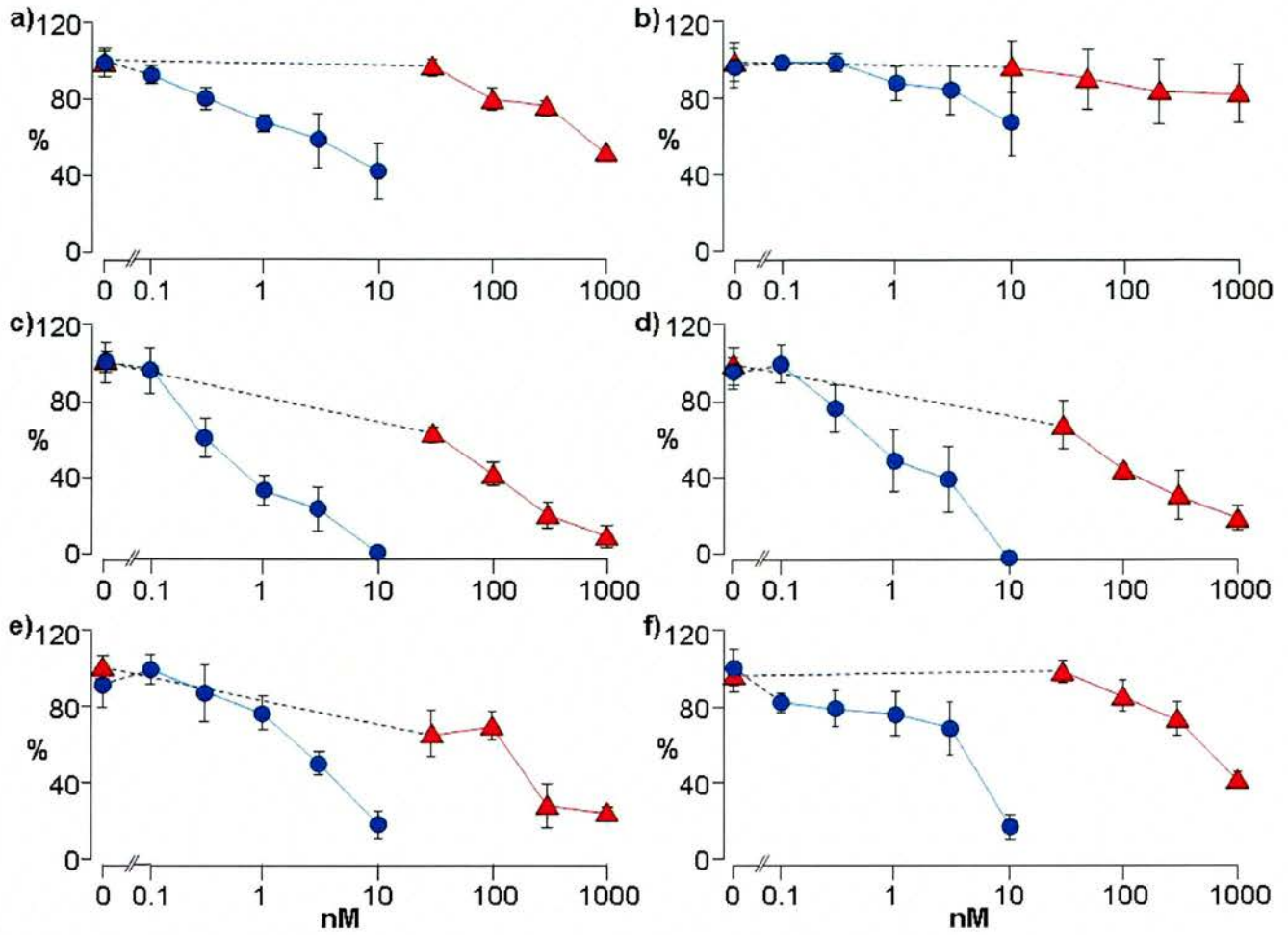


Figure 25: a composite figure accumulating all of the data from 12 animals comparing the effects of 4-DAMP (●) and AF-DX (▲) upon the response induced by 1000nM arecaidine. Data is expressed as a mean percentage of maximal response \pm 1 standard error of the mean, and represents data shown in figures 16 and 18. IF_{max} and F_{ss} are shown in figures a) and b) respectively. Underlying pressures initially (P_{init}) and at steady state (P_{ss}) are shown in c) and d) respectively. Figures e) and f) show the mean amplitudes during the initial phase and at steady state respectively.

3.5 Intrinsic characteristics of volume induced changes in augmented spontaneous phasic activity: the volume response

3.5.1 Overview

Intrinsic characteristics of the volume response with relation to volume increase, time of increase, and agonist concentration were assessed in addition to the effects on the response itself by hexamethonium, pancuronium, tolterodine, α/β methylene ATP, and purinergic receptor antagonists. Calculation of the instantaneous frequency and amplitude of the transients were calculated in a manner similar to that previously described. However, further characteristics of this specific response are described below. A typical example of a standard volume response is illustrated, Figure 26. Initially, regular transient contractions are seen and these are associated with a fixed baseline intravesical volume, typically in the region of 200 - 400 μ l. 2.0ml are added to the intravesical volume and this is associated with an increase in frequency and underlying pressure. Initially after the volume increase the frequency and underlying pressure is highest. Both reduce over time and settle at a steady state. After a set time, typically 500seconds, the intravesical volume is reduced by 2.0ml back to baseline volume. This results in a quiescent period where no transient contractions are seen which is followed by a gradual return in transient frequency and amplitude back to levels seen pre-volume increase.

Figure 27 shows a plot of the instantaneous frequency of the transients shown in Figure 26. It illustrates calculation of the 'baseline frequency', 'initial frequency', 'frequency at steady state', and tau values related to the length of the 'inhibitory phase'. Figure 26 also illustrates the initial phase of the volume response, the steady state, and the inhibitory phase.

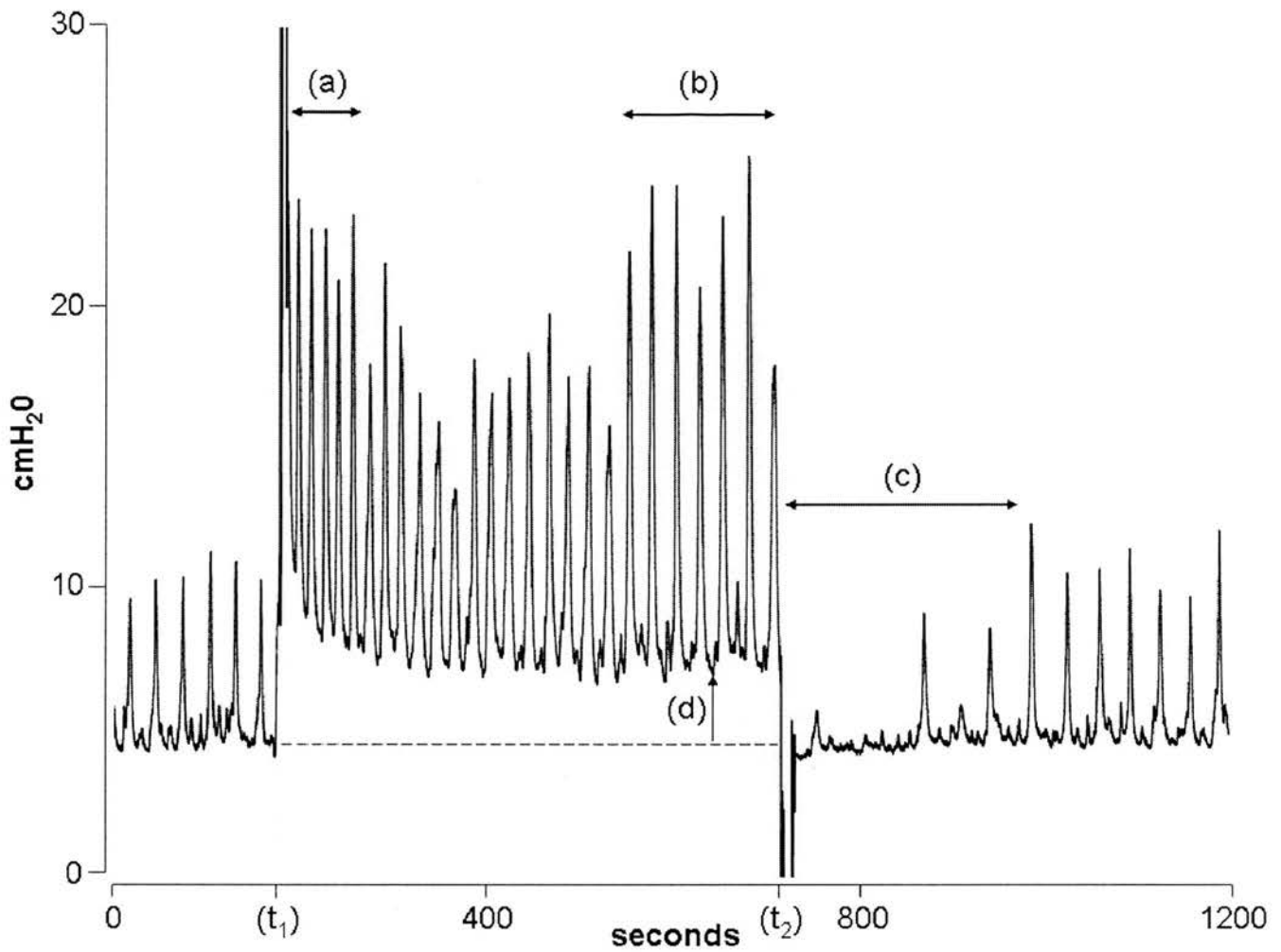


Figure 26: typical example of a 'volume response' performed in an isolated guinea pig bladder, with phasic activity augmented by 100nM arecaidine. t_1 represents the time point at which intravesical volume was increased, in this case by 2.0ml, and t_2 the time point where volume was reduced back to baseline volume. $(t_1 - t_2)$ is termed the 'duration of volume increase' and in this case is 500 seconds. The time interval taken to increase, or decrease, volume is typically < 10 seconds. (a) refers to the period immediately following the volume increase where there is an initial increase in frequency and underlying pressure followed by a gradual decrease. Qualitatively this area immediately succeeding the volume increase is known as the 'initial phase'. (b) refers to the area following a volume increase where the frequency, amplitude and underlying pressure of transient contractions remain relatively constant, termed 'steady state'. (c) refers to the time immediately following a volume decrease where a quiescent period is seen followed by a gradual return in activity, termed the 'inhibitory phase'. A clear demarcation of the end of the inhibitory phase is difficult; therefore, the point at which the frequency returns to 66.6% of the frequency prior to the volume increase is typically used in calculations; labelled the 'tau value'. (d) refers to the points used to calculate the increase in underlying pressure.

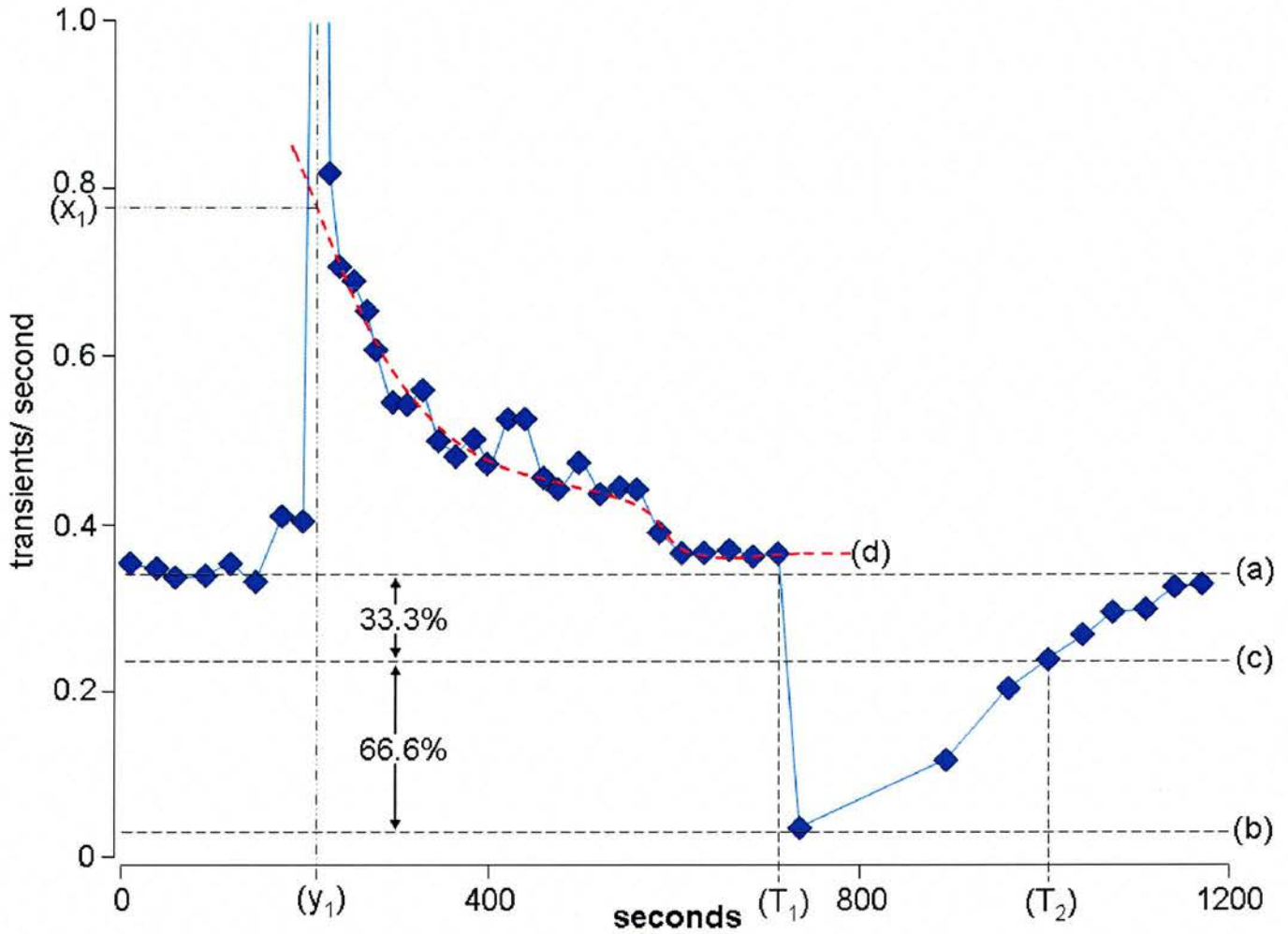


Figure 27: shows the instantaneous frequency of transients during the volume response illustrated in Figure 26. The red dashed line represents a line of best fit drawn during the period of increased volume. (y_1) represents a vertical axis drawn at the point of volume increase. Intersection of the line of best fit and (y_1) represents the frequency at the point of volume increase (IF_{init}), extrapolation of this intersection to the ordinate axis giving the value of IF_{init} , shown as (x_1). In this case IF_{init} equals 0.78 transients/second. (d) represents the frequency at steady state. (a) – (c) are used in the calculation of the tau value for the inhibitory phase; the point when frequency returns to 66.6% of its baseline, ‘pre-volume increase’ amount. (a) represents the baseline frequency prior to volume increase, note the frequency following volume decrease is returning to this level. (b) represents the lowest frequency following volume decrease. (c) represents an arbitrary line drawn 66.6% of the distance between (a) and (b). (T_1) represents the time at volume decrease, (T_2) is extrapolated from the intersection between the instantaneous frequency plot and (c); representing the point at which the frequency returns to 66.6% of baseline. The tau value for the inhibitory phase = ($T_2 - T_1$).

3.5.2 Definition and calculation of parameters specific to the ‘volume response’

- (i) *Pre-fill phase*: refers to the period prior to volume increase.
- (ii) *Initial phase*: a qualitative term referring to the time immediately succeeding volume increase that is associated with an increase in the instantaneous frequency of transients and rise in underlying pressure. Illustrated in Figure 26, (a).
- (iii) *Steady state*: refers to the period during a volume increase where transient frequency, amplitude and underlying pressure remain relatively constant, ((b), Figure 26).
- (iv) *Inhibitory phase*: refers to the time following volume decrease where a period of quiescence is followed by a gradual return in activity, ((c), Figure 26). For the purpose of quantitative analysis the *tau value of the inhibitory phase* is calculated, defined as the time taken following a volume decrease for the frequency to return to 66.6% of its pre-fill value. Illustrated Figure 27.
- (v) *pre-fill frequency* (F_{pre}): refers to the mean instantaneous frequency of the 4 transients prior to a volume increase.
- (vi) *initial frequency* (IF_{init}): refers to the instantaneous frequency of transients immediately at the time of a volume increase. This value is calculated from extrapolating a line of best fit of the transient

frequencies back to the time of volume increase, illustrated in Figure 27.

(vii) *frequency at steady state (F_{ss})*: refers to the instantaneous frequency of transients during steady state. Calculated from a line of best fit constructed manually by the researcher, illustrated Figure 27 (d).

(viii) *amplitude of pre-fill transients (A_{pre})*: refers to the amplitude of transients in the pre-fill phase. *Mean A_{pre}* is calculated for the 4 transient contractions immediately preceding the volume increase.

(ix) *amplitude during the initial phase (A_{init})*: refers to the amplitude of transients during the initial phase. *Mean A_{init}* is calculated from the first 4 transient contractions observed following a volume increase.

(x) *amplitude at steady state (A_{ss})*: refers to the amplitude of transients during the steady state phase. The last 4 transients of the steady state phase are used to calculate the *mean A_{ss}* .

(xi) *underlying pressure change (P_{shift})*: refers to the underlying pressure increase from that during the pre-fill phase to that seen at steady state, illustrated ((d), Figure 26). Similar to the underlying pressure at steady state, described prior to the previous results section, P_{shift} is calculated from a line of best fit added to a pressure/ time plot, incorporating the points of least amplitude, (Figure 9).

(xii) *duration of volume increase (t_{inc})*: refers to the time from which the volume increase has been completed to the time of completion of the volume decrease, illustrated by $(t_2 - t_1)$ Figure 26.

3.5.3 Differing effects of volume increase and decrease

The effects of three different changes in intravesical volumes were assessed; a typical example of these responses on the same organ preparation is shown in Figure 28. All volume responses were performed in the presence of 100nM arecaidine with the duration of volume increase lasting 500 seconds. Figure 28 a) shows a response to a volume increase and decrease of 2.0ml. In comparison Figure 28 b), shows the effects of an increase and decrease of 1.5ml. Figure 28 c) shows the response to a 2.0ml volume increase with a 1.5ml decrease. In all an increase in frequency both during the initial phase (IF_{init}) and at steady state (F_{ss}) can be seen. In addition, it is clear from both traces that the frequency during the initial phase is higher than that at steady state. It can also be noted that the length of the inhibitory phase reduces from trace a) through to c).

Figure 29 shows mean data \pm 1 standard error of the mean for five different preparations. Figure 29 a) shows the effects of the differing volumes on the inhibitory phase. This was most prolonged following a volume increase and decrease of 2.0ml, with a mean tau value of 212 ± 19 seconds. A volume increase and decrease of 1.5ml gave a shorter inhibitory phase, mean tau 157 ± 9 seconds. However, the shortest inhibitory phase was seen where the volume was increased by 2.0ml and only decreased by 1.5ml, leaving 0.5ml above the pre-fill baseline volume; mean tau value

118 \pm 9 seconds. A significant difference ($p<0.05$) was seen between the tau values for all three sets of volume responses, except for between an increase and decrease of 1.5ml (1.5_1.5ml) and an increase of 2.0ml and decrease of 1.5ml (2.0_1.5ml), (one way ANOVA, post hoc Tukey's).

Figures 23 b) –d) show the responses to volume increases of 1.5 and 2.0ml on underlying pressure (P_{shift}), frequency following volume increase (IF_{max}) and frequency at steady state (F_{ss}) respectively. All show a significant increase in these parameters after a 2.0ml increase compared to that of a 1.5ml increase. Table 8 shows the mean data \pm 1 standard error of the mean for the figures 23 b) – d).

	1.5ml vol increase	2.0ml vol increase	P value
Mean P_{shift} (cmH ₂ O) \pm 1 SEM	1.41 \pm 0.19	1.98 \pm 0.41*	0.048
Mean IF_{init} (trans/ sec) \pm 1 SEM	0.081 \pm 0.004	0.106 \pm 0.004*	0.014
Mean F_{ss} (trans/ sec) \pm 1 SEM	0.048 \pm 0.007	0.062 \pm 0.009*	0.005

Table 8: showing the mean effects of the two volume increases 1.5ml and 2.0ml upon the change in underlying pressure (P_{shift}), (cmH₂O), frequency following volume increase (IF_{init}) and at steady state (F_{ss}), (transients/ second). Data has been calculated from five bladders, (n=5). A statistically significant increase between the two volume responses ($p<0.05$) is illustrated by an asterix (*) with the respective p value shown in the third column (two tailed, student t-test).

Figure 30 shows the effects of the two volume increase, 1.5 and 2.0ml, upon the mean amplitude of transient contractions during the pre-fill, initial and steady state phases. As expected no difference exists between the amplitudes pre-fill. However, the amplitudes during both the initial phase and at steady state are significantly higher following a 1.5ml volume increase than for a 2.0ml increase. Mean A_{init} for a 1.5ml increase is 13.4 \pm 1.5 cmH₂O and for 2.0ml is 9.7 \pm 1.2 cmH₂O, ($p=0.021$). For the 1.5

and 2.0ml volume increases mean A_{ss} is 14.5 ± 1.1 and 12.6 ± 1.9 cmH₂O respectively, ($p=0.015$).

This data shows that the mechanisms involved in the generation of the inhibitory phase are related to both volume decrease as well as volume increase, since the inhibitory phase is longer after a volume increase and decrease of 2.0ml as opposed to an increase of 2.0ml and decrease of 1.5ml. Although not a significant difference, an increase and decrease of 1.5ml leads to a longer inhibitory phase than an increase of 2.0ml and decrease of 1.5ml. This may infer that it is not the amount by which the volume is altered that is important but the level down to which the bladder volume is reduced.

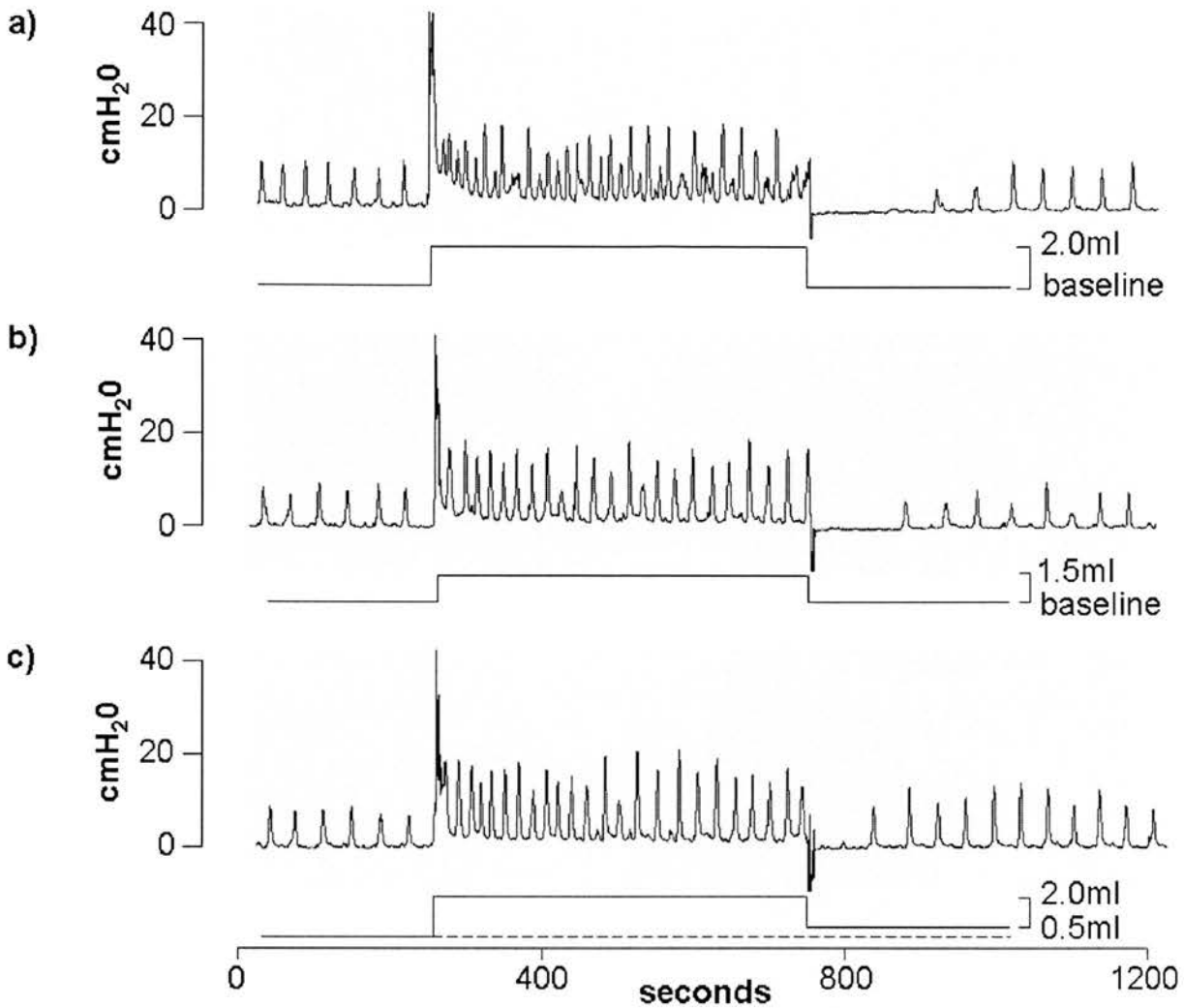


Figure 28: shows three volume responses, relating to differing volume changes, from the same isolated guinea pig bladder preparation. The amount by which intravesical volume was increased and then decreased is illustrated beneath each trace. Each response was performed in the presence of 100nM arecaidine and the volume increased for 500 seconds. a) underwent a 2.0ml increase followed by a 2.0ml decrease, b) a 1.5ml increase and 1.5ml decrease and c) a 2.0ml volume increase with a decrease of 1.5ml. Ordinate axes pressure (cmH₂O) and abscissa time (seconds). The pre-fill phases are similar for all three, however, in comparing a) and b) and increase in frequency can be seen initially and at steady state. Also, note a reduction in the inhibitory phase from a) through to c).

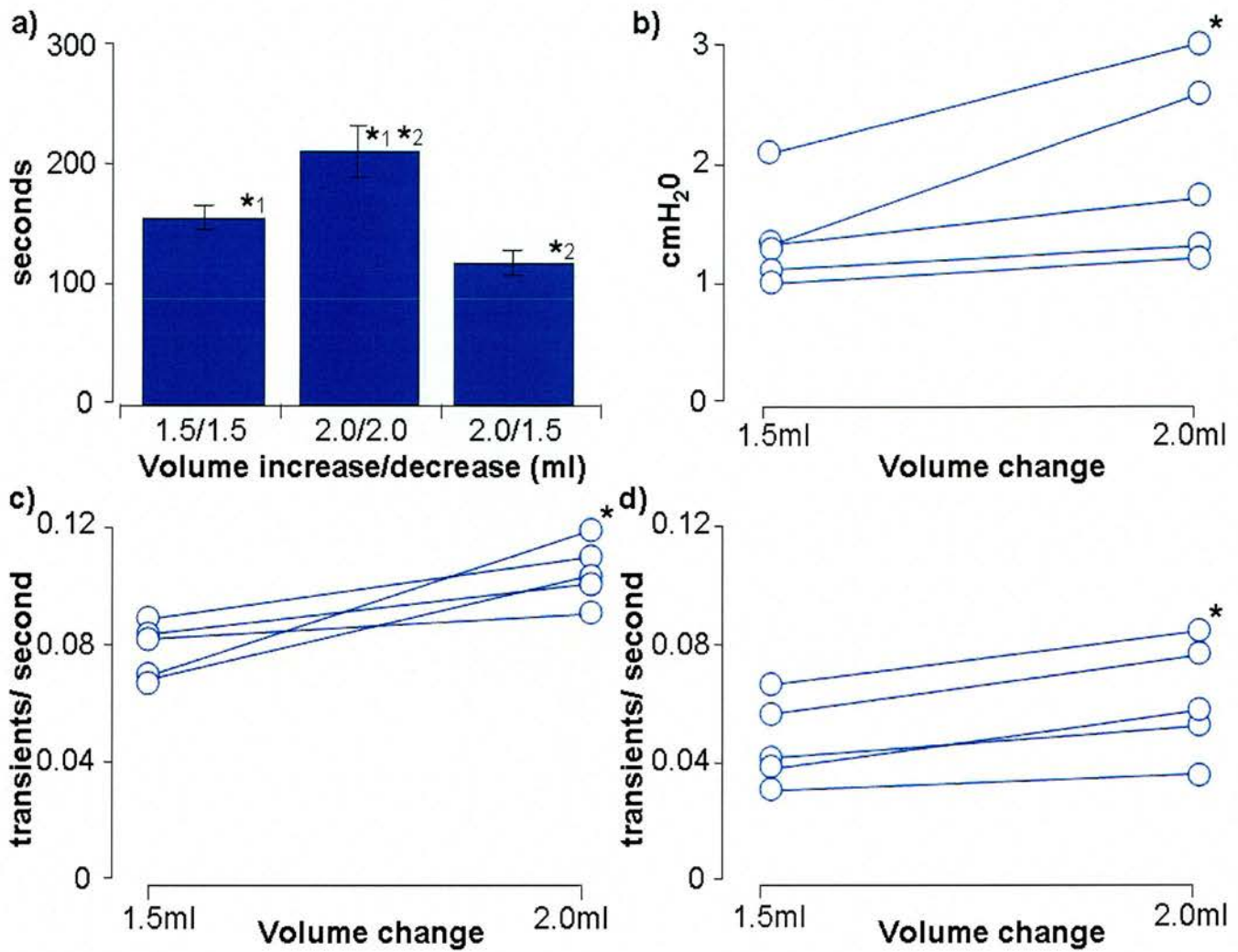


Figure 29: Data accumulated from 5 bladders illustrating the effects of different volumes changes on the 'volume response'. a) illustrates the effects of three combinations of volume changes on the tau value of the 'inhibitory phase'. Ordinate, tau value (seconds) and abscissa, volume increase/ decrease (ml). b) represents the change in underlying pressure (P_{shift}). Ordinate, pressure (cmH₂O) and abscissa, volume increase (ml). c) and d) illustrate the effects the two volume increases upon frequency following volume increase (IF_{init}) and at steady state (F_{ss}) respectively. Ordinates, frequency (transients/ second) and abscissae, volume increase (ml). Data points separated by a line in b) – d) represent results from the same bladder. Data in a) are shown as mean values ± 1 SEM (n=5). A significant difference between the data sets for each volume change b) – d) is indicated (*), ($p < 0.05$, two tailed student t-test). Significance between data points in (a) are illustrated, *¹ significant difference between 1.5_1.5ml and 2.0_2.0ml, *² significant difference between 2.0_2.0ml and 2.0_1.5ml ($p < 0.05$, one way ANOVA and post hoc Tukey's).

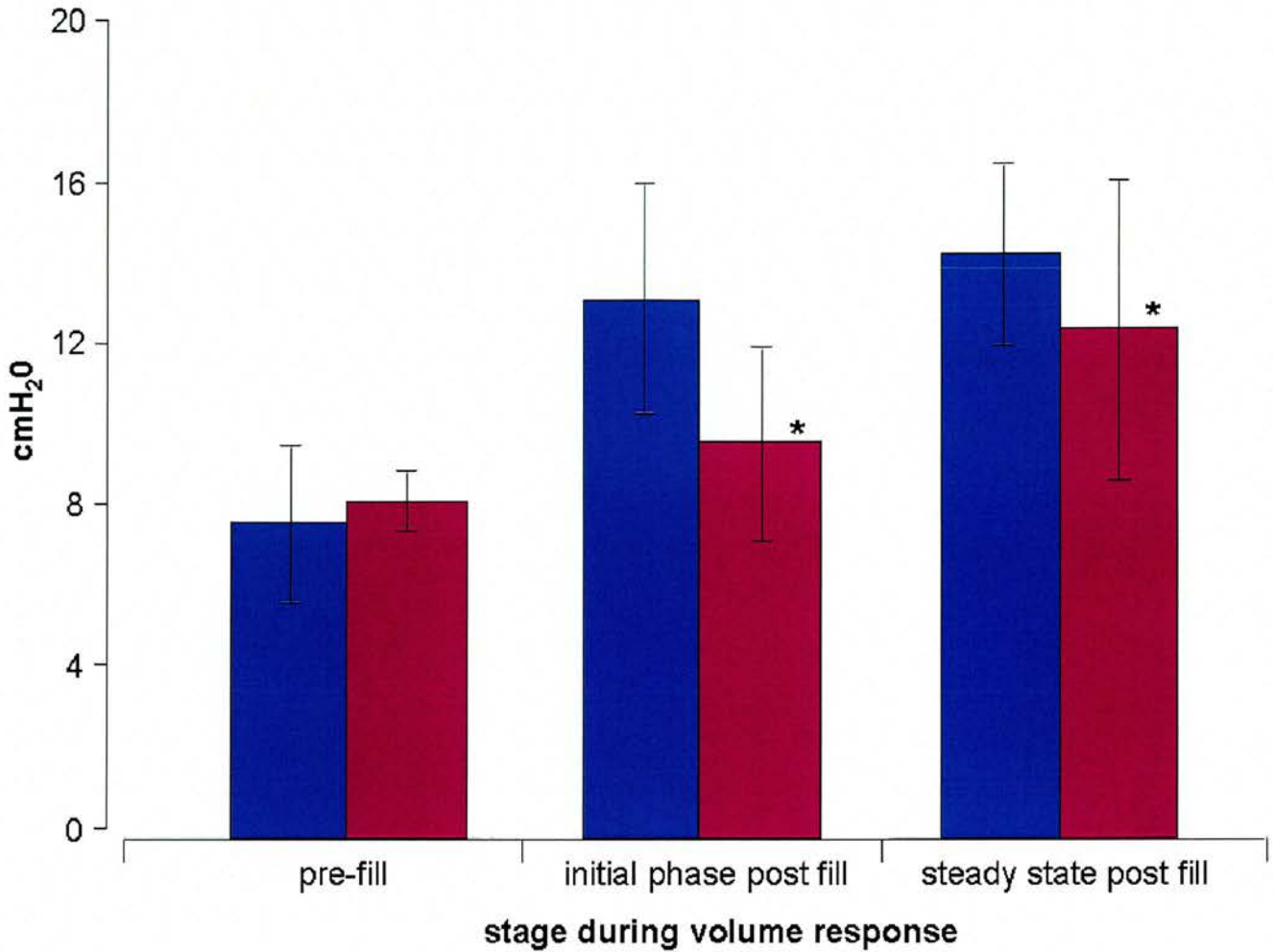


Figure 30: shows mean amplitudes for the two volume increases, 1.5ml (blue) and 2.0ml (maroon), during the three phases of the volume response; pre-fill, initial phase and steady state. Data was collected from five different isolated guinea pig bladder preparations, (n=5). Mean amplitudes are expressed ± 1 standard error of the mean. Ordinate, amplitude (cmH₂O) and abscissa, stages of the volume response. A significant difference between the two volume increases during a specific stage of the volume response is indicated (*), ($p < 0.05$, student t-test).

3.5.4 Effects of duration of volume increase

In the presence of 100nM arecaidine and using a volume increase/ decrease of 2.0ml the effects of increasing volume for a period of 10, 30, 100 and 500 seconds were assessed. A typical example of these different durations of volume increase performed on the same isolated bladder is shown in Figure 31. As the volumes both at baseline and following volume increase remain constant between the 4 experiments no differences in either the pre-fill or initial phase is seen between volume responses. What is apparent though is that the inhibitory phase increases along with the duration of volume increase.

Figure 32 shows the mean tau values of the inhibitory phase collected from 5 bladders. This illustrates that the inhibitory phase is dependent upon the duration of which intravesical volume is increased. A statistically significant difference is seen between all data points apart from the durations 10 and 30 seconds, and 100 and 500seconds.

This data suggests that a duration of volume increase of >30seconds is required in order to generate a significant inhibitory phase.

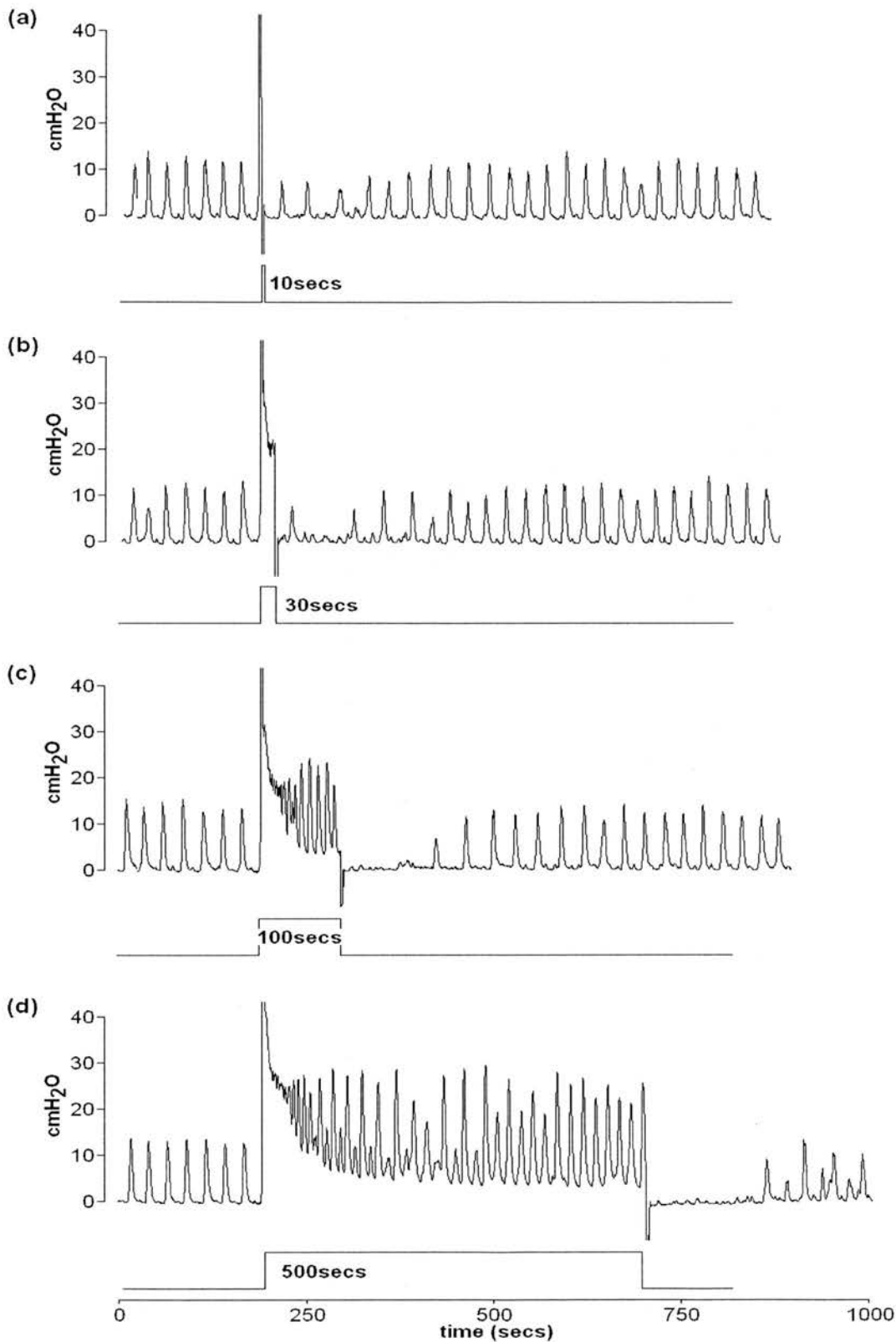


Figure 31: illustrates volume responses performed upon the same organ preparation; in the presence of 100nM arecaidine. Volumes were increased and decreased by 2.0ml for a period of 10, 30, 100 and 500 seconds respectively, (a-d). The duration of each volume increase is illustrated beneath each trace. Ordinate, pressure (cmH₂O) and abscissa, time (seconds).

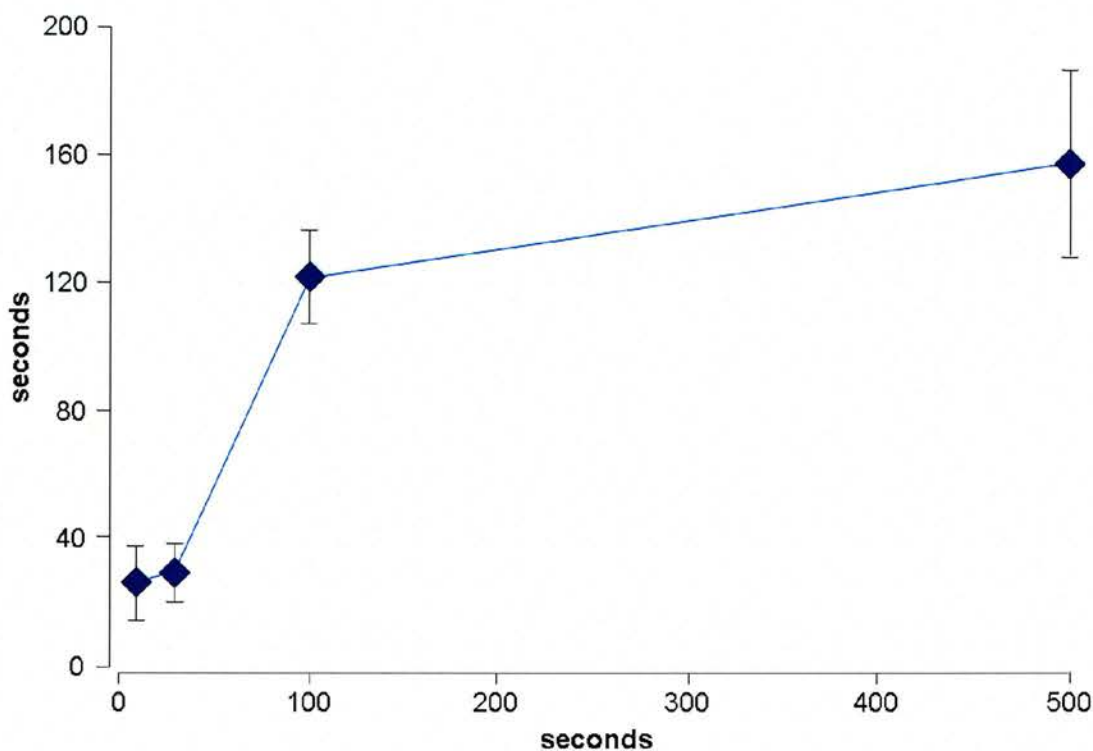


Figure 32: Data acquired from 5 bladders illustrating the effects of different durations of volume increase on the inhibitory phase. The abscissa represents the duration by which volume was increased (seconds). The ordinate axis represents the tau value for the inhibitory phase (seconds). Data points are shown as mean values ± 1 SEM (n=5). Apart from between the data points 10 and 30 seconds and 100 and 500 seconds a significant difference was seen between all the others, ($p<0.05$, one way ANOVA and post hoc Tukey's).

3.5.5 Effects of agonist concentration

The effects of the two arecaidine concentrations 100 and 300nM on the volume response were compared, with typical examples from the same bladder being shown in Figure 33. As shown previously increases in the frequency and amplitude of transient contractions is seen with increasing doses of arecaidine; illustrated again in these preparations by an increase in the frequency/ amplitude at baseline volume immediately prior to volume increase. Additionally, at the same dose, the frequency during the initial phase is higher than that at steady state. Comparing between the two doses, both IF_{init} and F_{ss} are higher in the presence of 300nM than 100nM arecaidine. However, the inhibitory phase is shorter in the presence of 300nM arecaidine.

Figure 34 shows mean data collected from 5 different bladders on the effects on the inhibitory phase (tau value), change in underlying pressure at steady state (P_{shift}), frequency following a volume increase (IF_{init}) and frequency at steady state (F_{ss}), a) – d) respectively. Table 9 summarises the mean data \pm 1 standard error of the mean shown in Figure 34. All volume responses were performed with duration of fill of 500seconds and a volume increase and decrease both of 2.0ml.

Figure 35 shows the effects of the two agonist concentrations, 100 and 300nM arecaidine, upon the mean amplitude of transient contractions during the pre-fill, initial and steady state phases. It shows that the amplitudes of transient contractions are larger with a higher dose of arecaidine, 300nM, during the pre-fill stage, which has essentially been highlighted previously in the 'effects of arecaidine/ carbachol'. However, the amplitudes during the initial phase become smaller with 300nM. But, during the steady state phase the amplitudes become larger again with 300nM. Mean A_{pre} with 100nM

arecaidine was 6.5 ± 1.2 cmH₂O and for 300nM arecaidine was 13.4 ± 0.7 cmH₂O, ($p=0.0005$). Mean A_{init} in the presence of 100 and 300nM were 9.5 ± 0.7 and 6.3 ± 0.9 cmH₂O respectively, ($p=0.014$). For 100 and 300nM respectively, mean A_{ss} was 14.3 ± 1.1 and 16.4 ± 0.8 cmH₂O, ($p=0.01$).

	100nM	300nM	P value
Mean tau value (seconds) \pm 1 SEM	236 ± 47	$178 \pm 30^*$	0.007
Mean P_{shift} (cmH ₂ O) \pm 1 SEM	2.8 ± 0.6	$3.7 \pm 0.5^*$	0.017
Mean IF_{init} (trans/sec) \pm 1 SEM	0.091 ± 0.014	$0.143 \pm 0.015^*$	0.018
Mean F_{ss} (trans/sec) \pm 1 SEM	0.059 ± 0.008	$0.075 \pm 0.01^*$	0.021

Table 9: summarises the mean data \pm 1 SEM from Figure 34, comparing the effects of two concentrations of arecaidine, 100 and 300nM, on the volume response. Data has been calculated from five bladders, ($n=5$). A statistically significant difference between the two agonist concentrations ($p<0.05$) is illustrated by an asterix (*) with the respective p value shown in the third column (two tailed student t-test).

This data shows that the addition of an increased dose of arecaidine reduces the inhibitory phase suggesting that the mechanisms involved in the return of spontaneous activity following volume induced inhibition are in part cholinergically mediated.

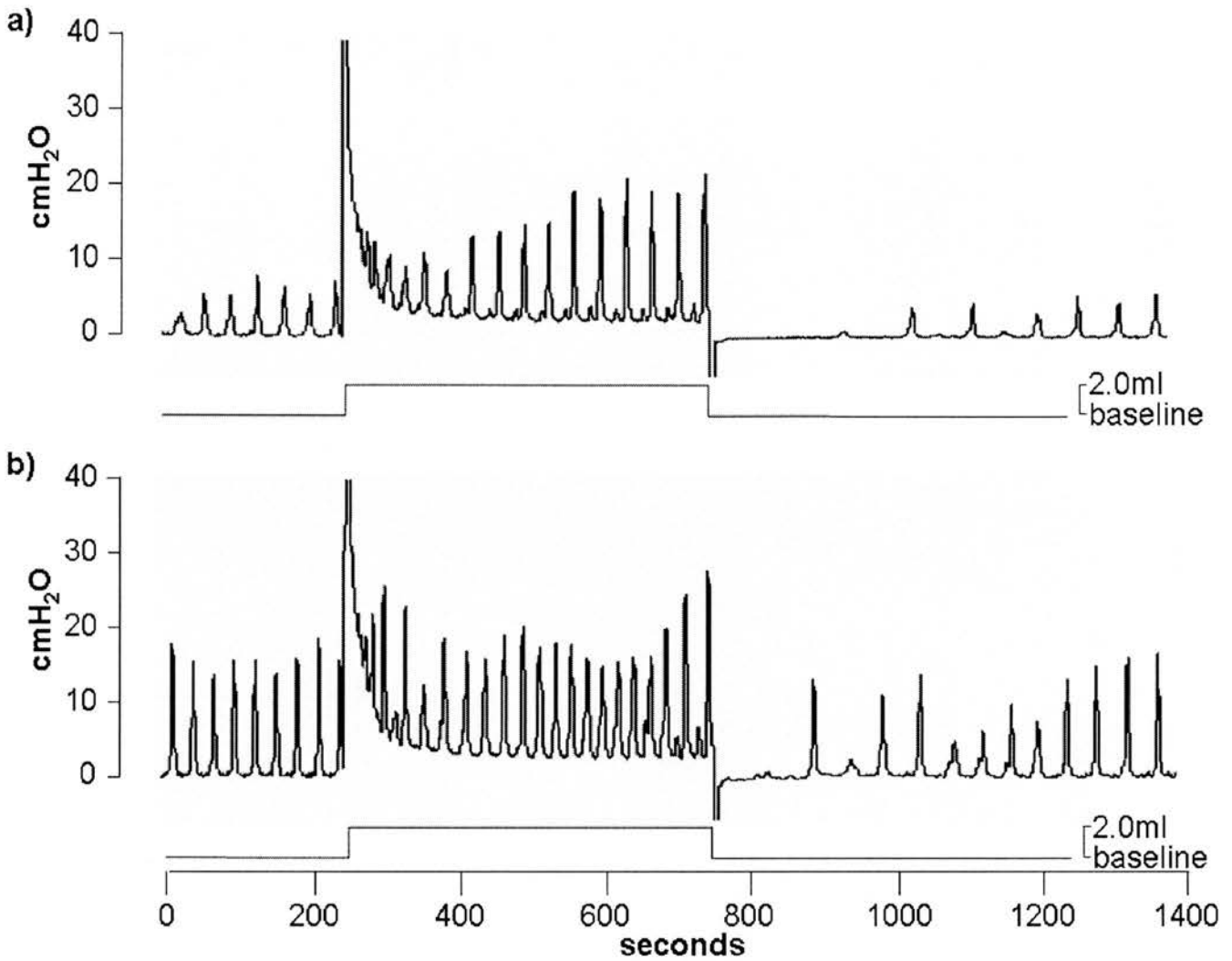


Figure 33: Typical traces of volume responses performed in the presence of 100nM and 300nM arecaidine, a) and b) respectively. The amounts by which the intravesical volumes were changed are illustrated beneath each trace, in addition to the duration of volume increase. In these cases volume changes were 2.0ml, lasting for 500seconds. Ordinates represent pressure (cmH₂O) and the abscissa time (seconds). Note, the increased frequency in all three phases, (pre-fill, initial, steady state), in the presence of 300nM arecaidine, b), compared to 100nM, a). Also, note the reduction in the inhibitory phase in the presence of 300nM arecaidine, b).

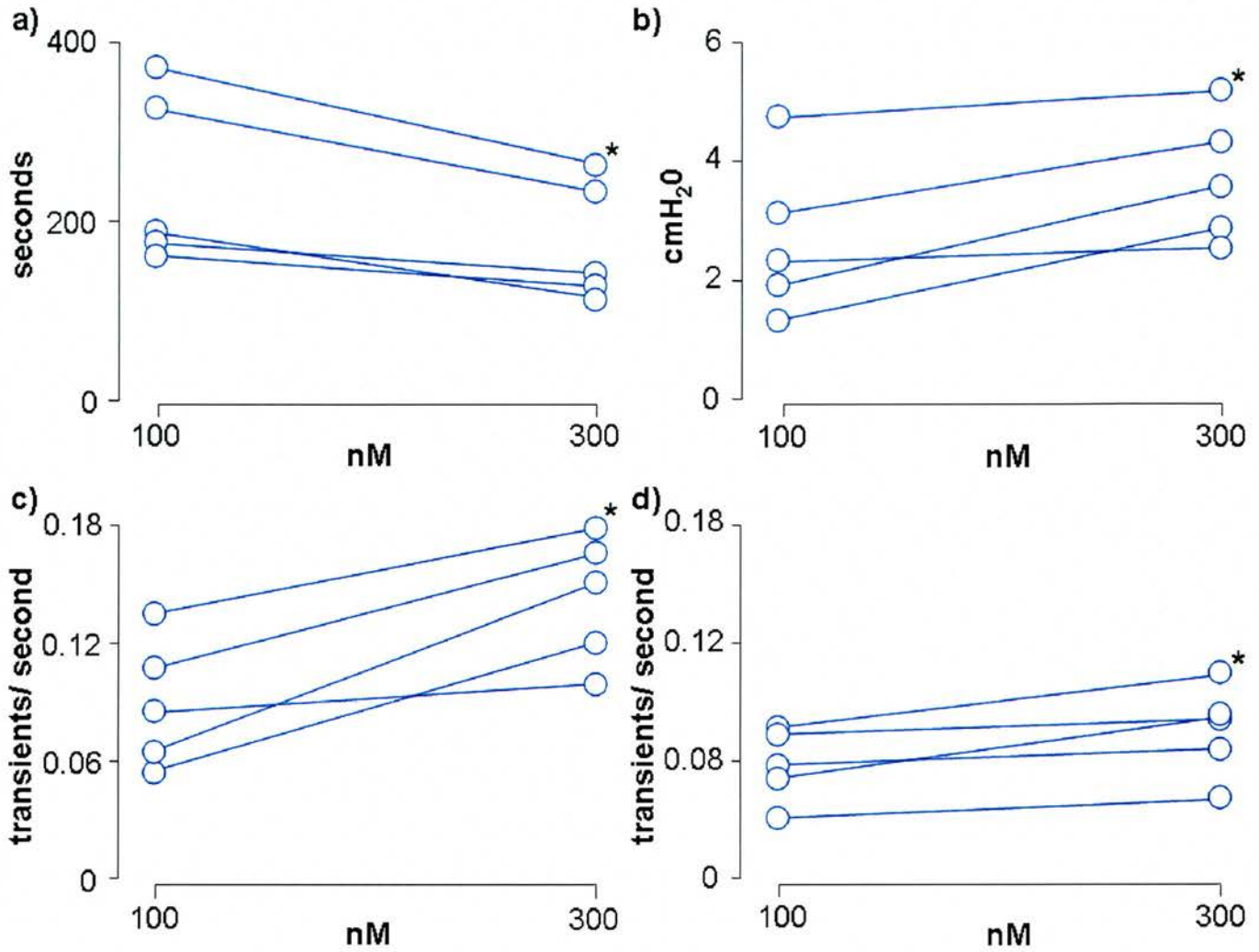


Figure 34: Data collected from five different isolated bladder preparations on the effects on the volume response in the presence of 100nM and 300nM arecaidine. Data points connected by a line represent results collected from the same bladder preparation. a) illustrates the effects of the two arecaidine doses on the inhibitory phase; ordinate axis, tau value for the inhibitory phase (seconds). b) represents the effects upon change in underlying pressure (P_{shift}); ordinate axis, pressure (cmH₂O). c) and d) represent the effects upon frequency following volume increase (IF_{init}) and frequency at steady state (F_{ss}) respectively; for both ordinate axis, instantaneous frequency of transients (transients/ second). In all four panels the abscissae relate to the two doses of arecaidine in which the volume response was performed (nM). A significant difference between the effects of the two concentrations is indicated (*), ($p < 0.05$, two tailed student t-test).

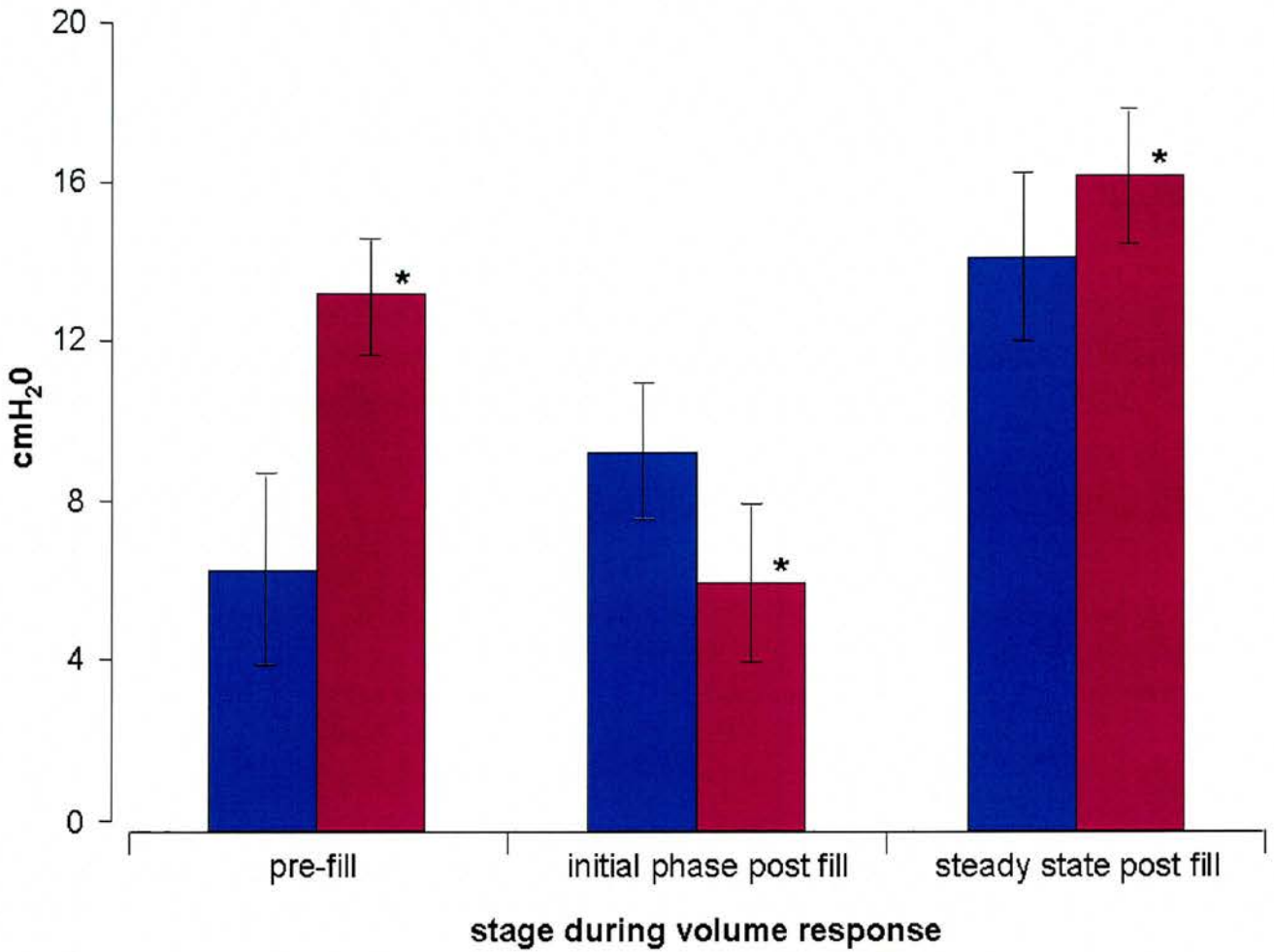


Figure 35: Mean amplitudes for three phases of the volume response performed in the presence of 100nM (blue) and 300nM arecaidine (maroon). Data was collected from five different isolated guinea pig bladder preparations, (n=5). Mean amplitudes are expressed ± 1 standard error of the mean. Ordinate, amplitude (cmH₂O) and abscissa, stages of the volume response. A significant difference between the two arecaidine concentrations during a specific stage of the volume response is indicated (*), (p<0.05, two tailed student t-test).

3.6 Effects of tolterodine upon the volume response

Tolterodine is an orally administered anti-muscarinic drug commonly used in the treatment of OAB. It is a competitive muscarinic antagonist selective for M_3 receptors $> M_2$. Its effects on the volume response in the isolated guinea pig bladder were assessed with a typical example of the responses to increasing doses illustrated in Figure 36. These 4 traces highlight a clear reduction in the amplitude of transient contractions at higher doses, with a slight reduction in frequency both initially and at steady state. However, the inhibitory phase seems to be obviously prolonged from 10nM upwards. Figure 37 illustrates data collected from 4 different guinea pig bladders showing the effects of tolterodine upon the inhibitory phase, change in underlying pressure (P_{shift}), frequency following volume increase (IF_{max}) and at steady state (F_{ss}); panels a) to d) respectively. This data is also summarised in table 10.

	Control	10nM	30nM	100nM
Mean tau (seconds)	187 ± 20	221 ± 26 (0.39)	260 ± 22 (0.059)	$400 \pm 14^*$ (0.003)
Mean P_{shift} (cmH ₂ O)	1.56 ± 0.5	1.1 ± 0.3 (0.15)	1.0 ± 0.42 (0.16)	0.84 ± 0.9 (0.22)
Mean IF_{max} (trans/ sec)	0.074 ± 0.006	0.065 ± 0.008 (0.17)	0.062 ± 0.005 (0.072)	$0.058 \pm 0.003^*$ (0.046)
Mean F_{ss} (trans/ sec)	0.049 ± 0.005	0.049 ± 0.005 (0.62)	0.046 ± 0.006 (0.068)	0.043 ± 0.004 (0.055)

Table 10: summarises the mean data ± 1 SEM illustrated in Figure 36; comparing the effects of increasing doses of tolterodine upon the volume response. Data has been calculated from four bladders, (n=4). A statistically significant difference between doses and the control is illustrated by an asterix (*, $p < 0.05$). Respective p values referring to the statistical difference between a dose and its control are shown in brackets, (one way ANOVA and post hoc Tukey's).

Figure 38 illustrates the effects of tolterodine upon the amplitude of transient contractions during the different stages of the volume response.

This data illustrates that at higher doses of tolterodine, 100nM, the inhibitory phase is significantly prolonged and the initial frequency is significantly reduced. Since tolterodine has been shown to be clinically effective in the treatment of OAB it can be speculated that some of these beneficial effects are due to effects upon mechanisms involved in these specific responses.

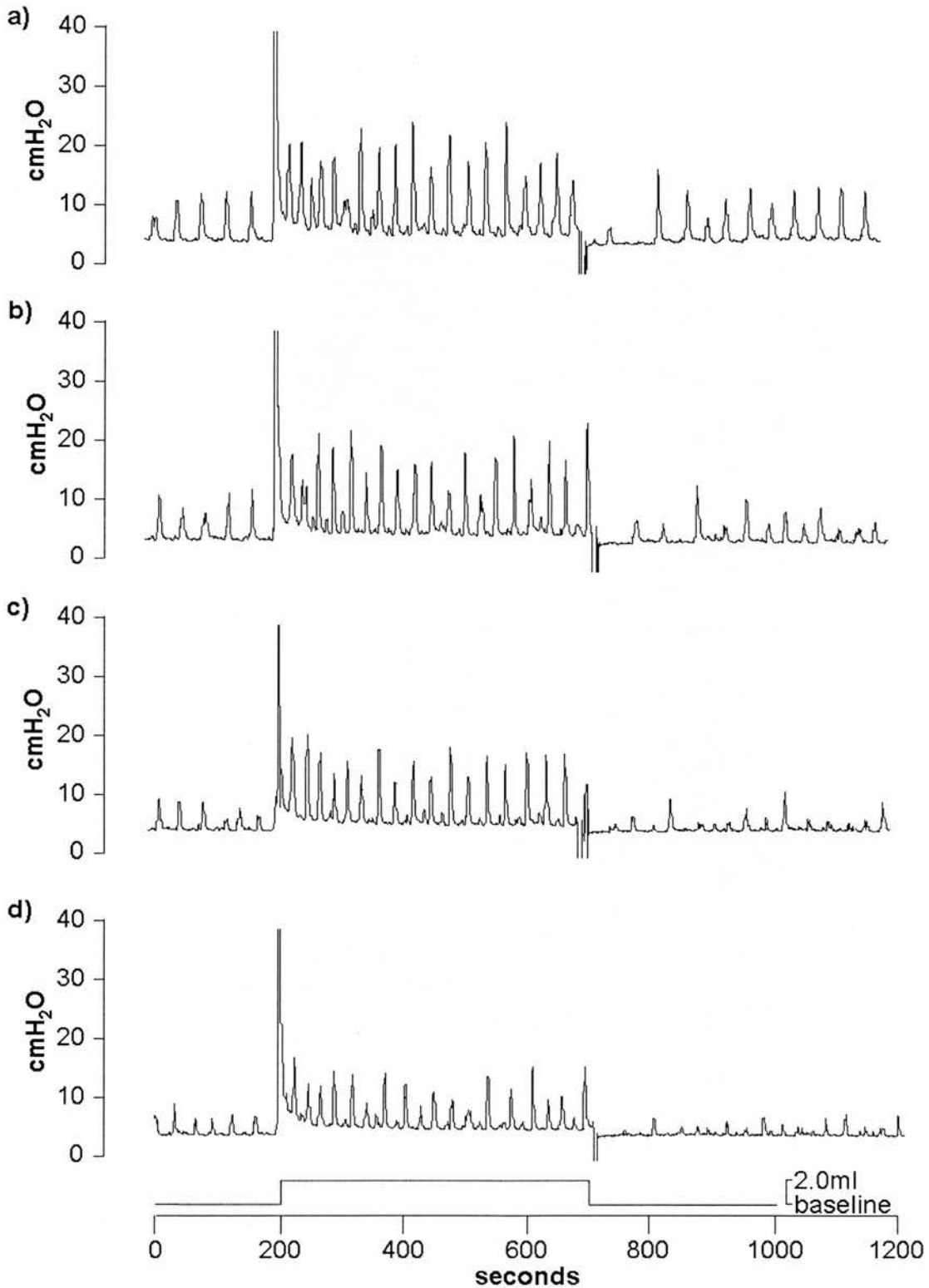


Figure 36: Typical traces, from the same isolated bladder preparation, showing the effects of increasing doses of tolterodine upon the volume response. a) control, b) 10nM, c) 30nM and d) 100nM. In all traces responses were elicited in the presence of 100nM arecaidine with volume increased/ decreased by 2.0ml and the duration of fill lasting 500seconds. Ordinates represent pressure (cmH₂O) and the abscissa time (seconds). Tolterodine was added directly to the bathing solution prior to the start of the recordings above.

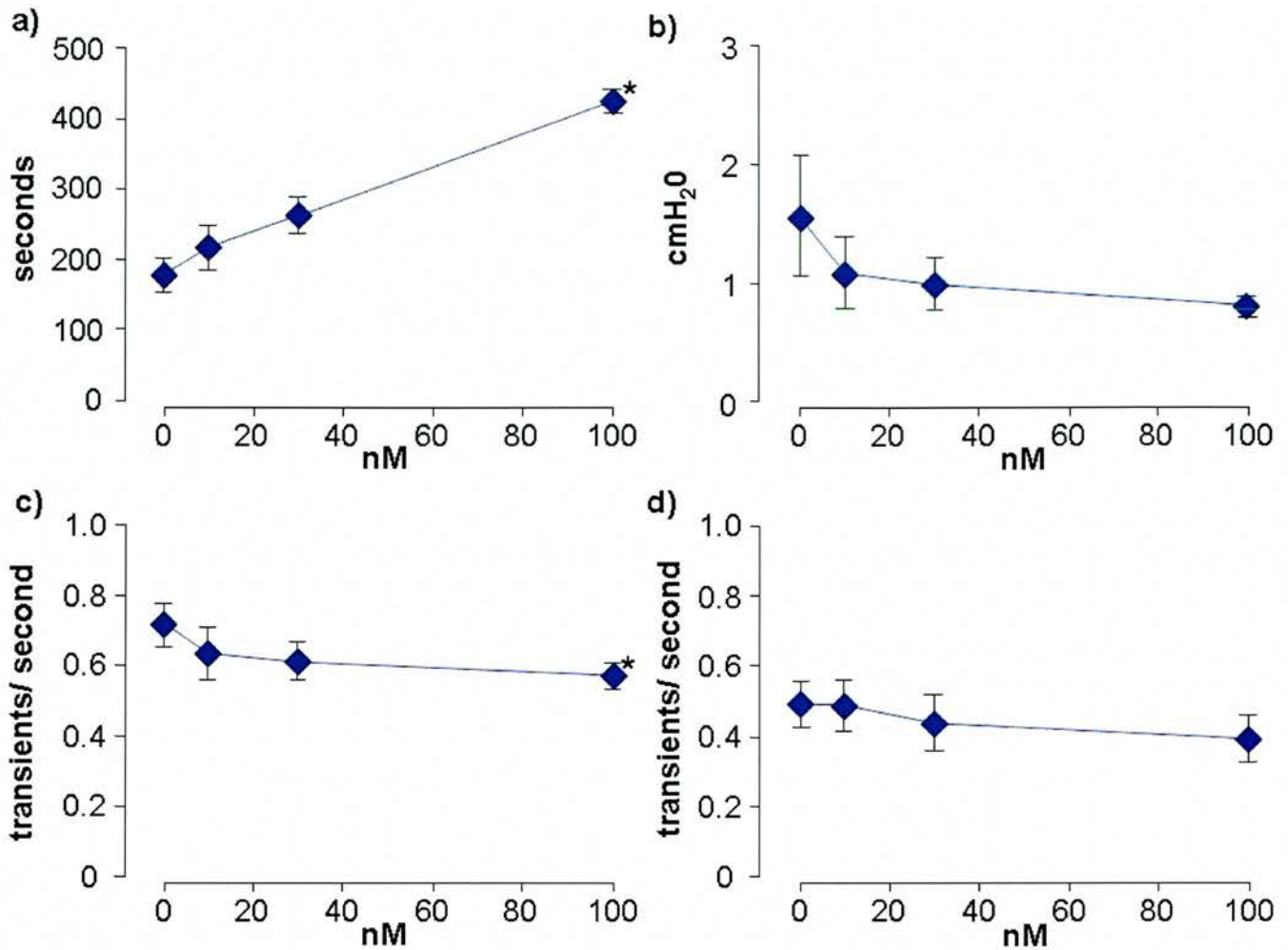


Figure 37: Data collected from 4 bladders illustrating the effects of increasing doses of tolterodine upon the volume response. a) effects on the tau value of the inhibitory phase. Ordinate, tau value (seconds). b) represents change in underlying pressure (P_{shift}). Ordinate, pressure (cmH₂O). c) and d) illustrate the effects of tolterodine upon frequency following volume increase (IF_{init}) and at steady state (F_{ss}) respectively. Ordinates for c) and d), frequency (transients/ second). For all panels the abscissa represents the dose of tolterodine added to the preparation (nM). Data points are shown as mean values \pm 1 SEM (n=4). A significant difference between the data points compared to control is indicated (*), ($p < 0.05$, one way ANOVA and post hoc Tukey's).

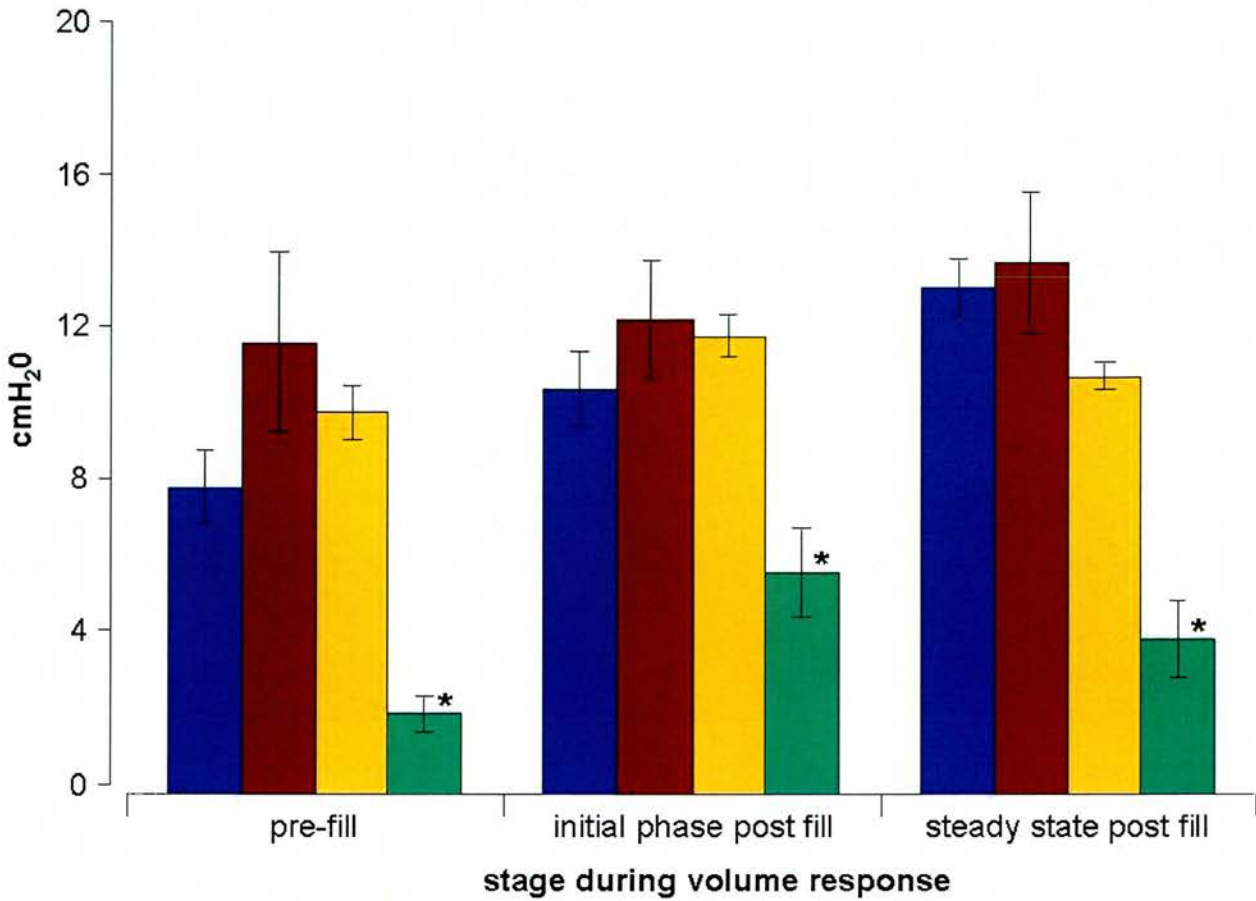


Figure 38: Data from 4 bladders showing the effects of increasing doses of tolterodine upon the amplitude of transient contractions during the pre-fill, initial and steady state phases. Blue column represents the control response for each stage where no tolterodine was added. Maroon, in the presence of 10nM tolterodine; gold, 30nM; and green, 100nM. Mean amplitudes are expressed ± 1 standard error of the mean, (n=4). Ordinate, amplitude (cmH₂O) and abscissa, stages of the volume response. A significant difference between doses and the control during a specific stage of the volume response is indicated (*), (p<0.05, one way ANOVA and post hoc Tukey's).

3.7 Effects of the nicotinic receptor antagonists, Hexamethonium and Pancuronium, on the volume response

The effects of hexamethonium, an antagonist specific to ganglionic nicotinic receptors, and pancuronium, an antagonist selective for nicotinic receptors typically found on skeletal muscle, were assessed on the volume response in the isolated guinea pig bladder. Both were found to have an effect during the volume response following a volume increase, however, only pancuronium exhibited any effect on underlying augmented activity, i.e., activity during the pre-fill stage. In the first instance the effects of the individual antagonists upon the volume response will be described followed by a comparison of their effects during the pre-fill phase.

3.7.1 Effects of hexamethonium on the volume response

Hexamethonium was found to significantly prolong the inhibitory phase at a dose of $3\mu\text{M}$. However, no significant effect was seen on either the frequency following volume increase (IF_{init}), frequency at steady state (F_{ss}) or change in underlying pressure (P_{shift}) when compared to control values. A typical example of traces collected from the same organ preparation is illustrated in Figure 39. These volume responses were performed in the presence of 100nM arecaidine, with a 2.5ml increase in intravesical volume lasting for 500seconds.

Mean data collected from 5 different bladders are shown in Figure 40. Figure 40a) illustrates the increase in tau values for the inhibitory phase associated with hexamethonium. A dose dependent response was not seen as the inhibitory phase was more prolonged in the presence of $3\mu\text{M}$ rather than $10\mu\text{M}$. The mean tau value with no hexamethonium added, (control), was 239 ± 23 seconds; mean \pm SEM. In the presence of $3\mu\text{M}$ hexamethonium mean tau value was 346 ± 35 seconds, with a statistically

significant increase being seen compared with control, ($p=0.025$). For $10\mu\text{M}$ mean tau was 320 ± 79 seconds; ($p=0.054$). The change in underlying pressure from pre-fill to steady state, P_{shift} , is illustrated in Figure 40b). No significant reductions are seen at either dose. Figure 40c) and d) illustrate that hexamethonium has no effect upon either the instantaneous frequency of transient contractions following volume increase (IF_{init}) or the frequency at steady state F_{ss} respectively.

The effects of hexamethonium upon the amplitude of transient contractions are illustrated in Figure 41. It can be seen that the amplitude of contractions increase through the three stages of the volume response, pre-fill, initial and steady state. However, no statistical difference can be seen between control and either dose of hexamethonium during any single stage.

This data shows that hexamethonium at a dose of $3\mu\text{M}$ significantly prolongs the inhibitory phase. This would suggest that nicotinic receptors, more specifically those related to ganglia, are involved in the return of activity following volume induced inhibition. Since there little response on the other parameters is seen it would seem that these receptors are not involved in the regulation of the frequency or the generation of spontaneous activity.

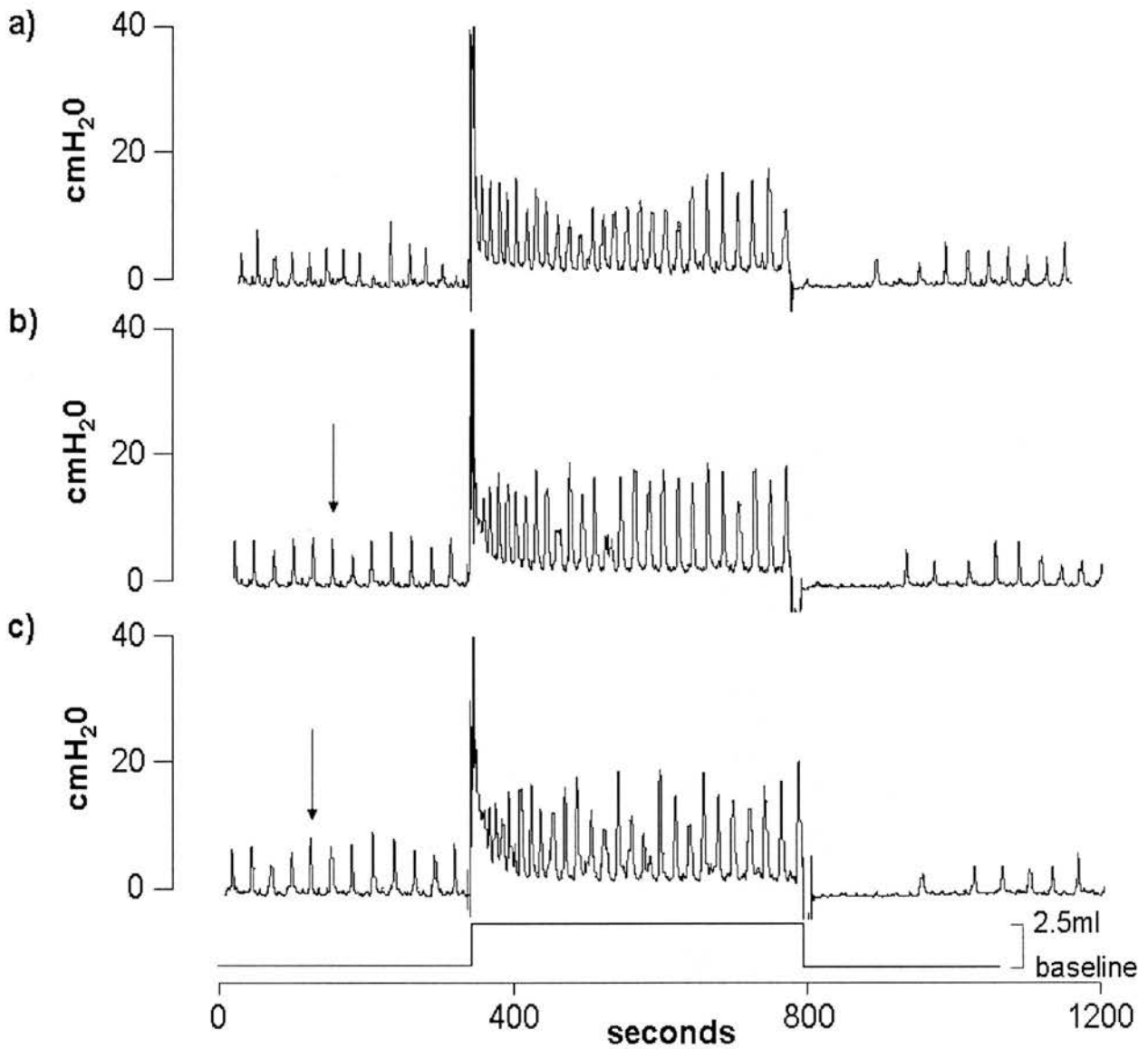


Figure 39: Typical traces showing the effects of 3 and 10 μ M hexamethonium upon the volume response in the isolated guinea pig bladder; taken from the same organ preparation. Volume responses were performed in the presence of 100nM arecaidine, with a volume increase of 2.5ml lasting for 500 seconds. a) control response, no hexamethonium added. b) and c) illustrate responses in the presence of 3 and 10 μ M hexamethonium respectively. Arrows indicate the point at which hexamethonium was added to the Tyrode's solution bathing the abluminal surface of the bladder. Ordinates represent pressure (cmH₂O) and the abscissa time (seconds). The volume and duration of volume increase is illustrated above the abscissa.

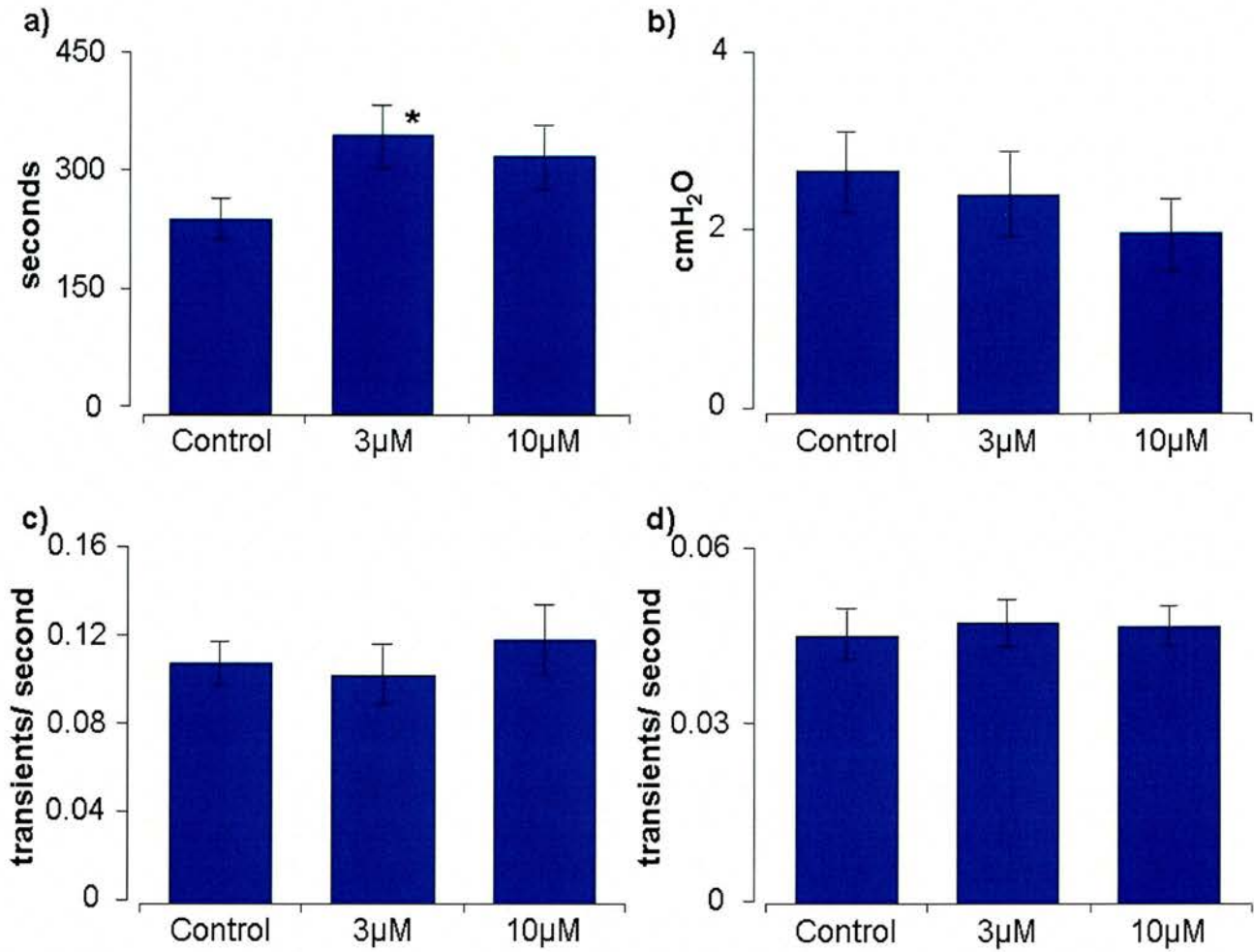


Figure 40: Data collected from 5 bladders illustrating the effects of two doses of hexamethonium, 3 and 10 μM , on the volume response. a) illustrates the effects upon the tau value of the inhibitory phase. Ordinate, tau value (seconds). b) represents the change in underlying pressure (P_{shift}). Ordinate, pressure (cmH₂O). c) and d) illustrate the effects upon frequency following volume increase (IF_{init}) and at steady state (F_{ss}) respectively. Ordinates, frequency (transients/ second). The abscissa in all panels represents the dose of hexamethonium in which the volume response was performed (μM). Data are shown as mean values \pm 1 SEM ($n=5$). A significant difference between doses compared to control is indicated (*), ($p<0.05$, one way ANOVA and post hoc Tukey's).

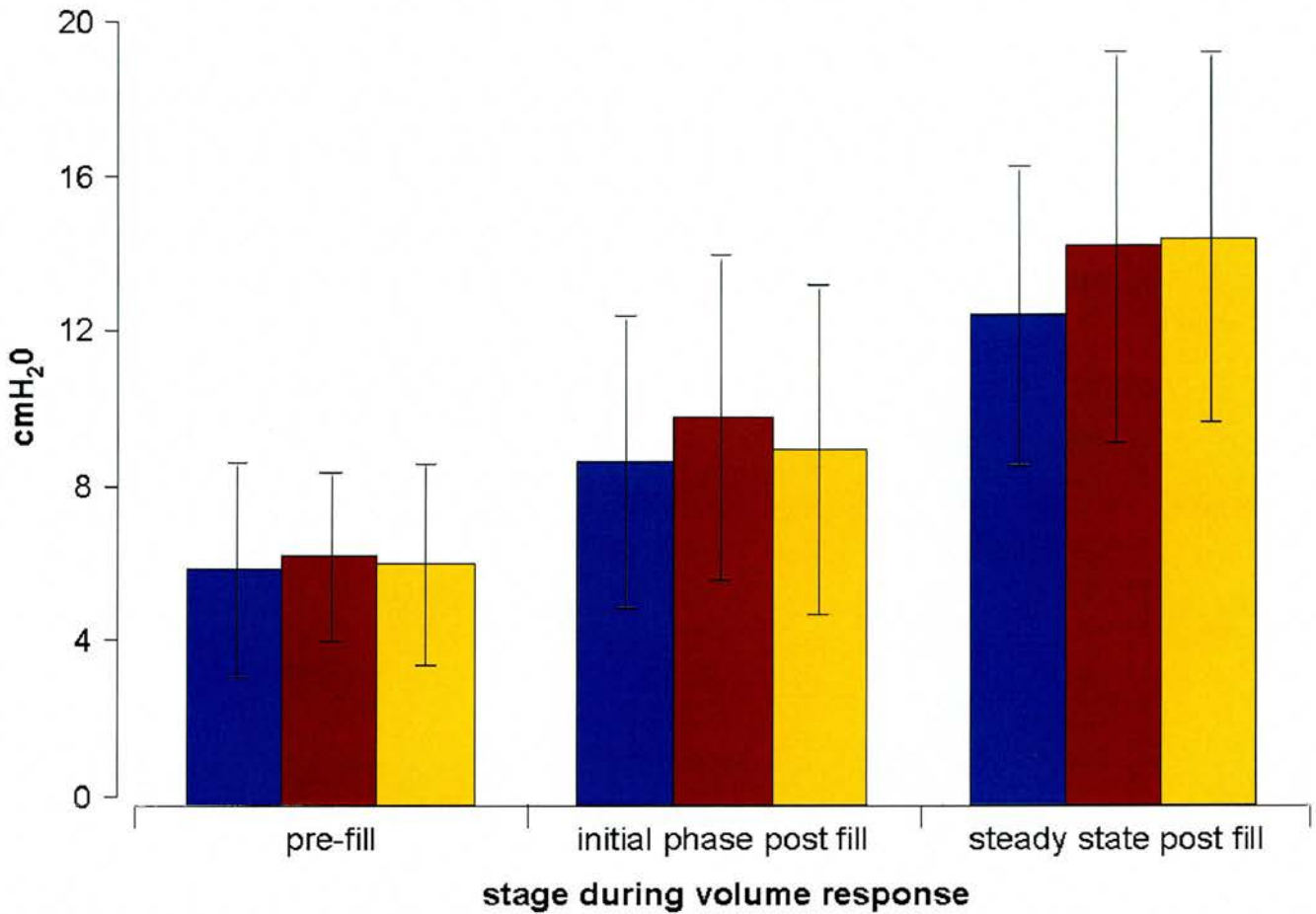


Figure 41: Data from 5 bladders showing the effects of 3 and 10 µM hexamethonium upon the amplitude of transient contractions during the pre-fill, initial and steady state phases. Blue column represents the control response for each stage where no hexamethonium was added. Maroon, 3 µM hexamethonium; gold, 10 µM hexamethonium. Mean amplitudes are expressed ± 1 standard error of the mean, (n=5). Ordinate, amplitude (cmH₂O) and abscissa, stages of the volume response. No significant differences between doses and the respective control were seen, (p<0.05, one way ANOVA).

3.7.2 Effects of pancuronium bromide on the volume response

In contrast to hexamethonium, pancuronium exerted effects on all the stages of the volume response. A typical example of responses obtained from the same bladder preparation following the addition of 3 and 10 μ M pancuronium are illustrated in Figure 42. As with hexamethonium, these volume responses were performed in the presence of 100nM arecaidine with intravesical volume increased by 2.5ml for a period of 500 seconds.

The mean data from the six bladders used to study the effects of pancuronium are described in Figure 43. Figure 43a) illustrates the increase in tau values for the inhibitory phase associated with pancuronium. The mean control tau value in the absence of pancuronium was 232 ± 35 seconds. In the presence of 3 μ M pancuronium mean tau was 307 ± 32 seconds, which was not significantly increased compared to control. However, at 10 μ M mean tau was significantly increased at 410 ± 67 seconds, compared to control ($p=0.016$). Figure 43b) illustrates that no significant change is seen in the change in underlying basal pressure (P_{shift}) at either dose. The frequencies during the initial phase were noted to be significantly reduced by both doses of pancuronium. However, during steady state a decrease in frequency was only seen at 10 μ M. Figure 43c) illustrates the effects upon frequency following volume increase (IF_{init}). Mean IF_{init} for control, 3 and 10 μ M are 0.130 ± 0.02 transients/ second, 0.082 ± 0.01 ($p=0.03$, compared to control) and 0.078 ± 0.01 ($p=0.04$) respectively. The effects upon frequency at steady state are demonstrated in Figure 43d). Mean F_{ss} for control is 0.004 ± 0.01 transients/ second. In the presence of 3 μ M pancuronium although a reduction was seen this was not significant, mean 0.036 ± 0.006 transients/ second. Mean F_{ss} with 10 μ M pancuronium was 0.034 ± 0.006 , ($p=0.048$).

Figure 44 demonstrates the effects of pancuronium upon the amplitude of transient contractions. It can be seen that the amplitude of contractions are significantly reduced compared to control values during the pre-fill and steady state phases. However, no significant difference was seen during the initial phase. Also, no significant difference exists between the two doses in any of the three stages.

This data reveals that pancuronium significantly increases the inhibitory phase at a dose of 10 μ M in addition to significantly reducing the frequency at steady state. At doses of 3 and 10 μ M frequency both pre volume increase and immediately following volume increase are significantly reduced. This again would suggest a role for nicotinic receptors, similar in nature to those found on skeletal muscle, in the modulation of the inhibitory phase and the generation of the frequency of spontaneous contractions. (It is acknowledged that pancuronium can have non specific anti-muscarinic (M_2 / M_3) effects however, the effects of pancuronium are different to those seen following tolterodine, 4-DAMP and AF-DX).

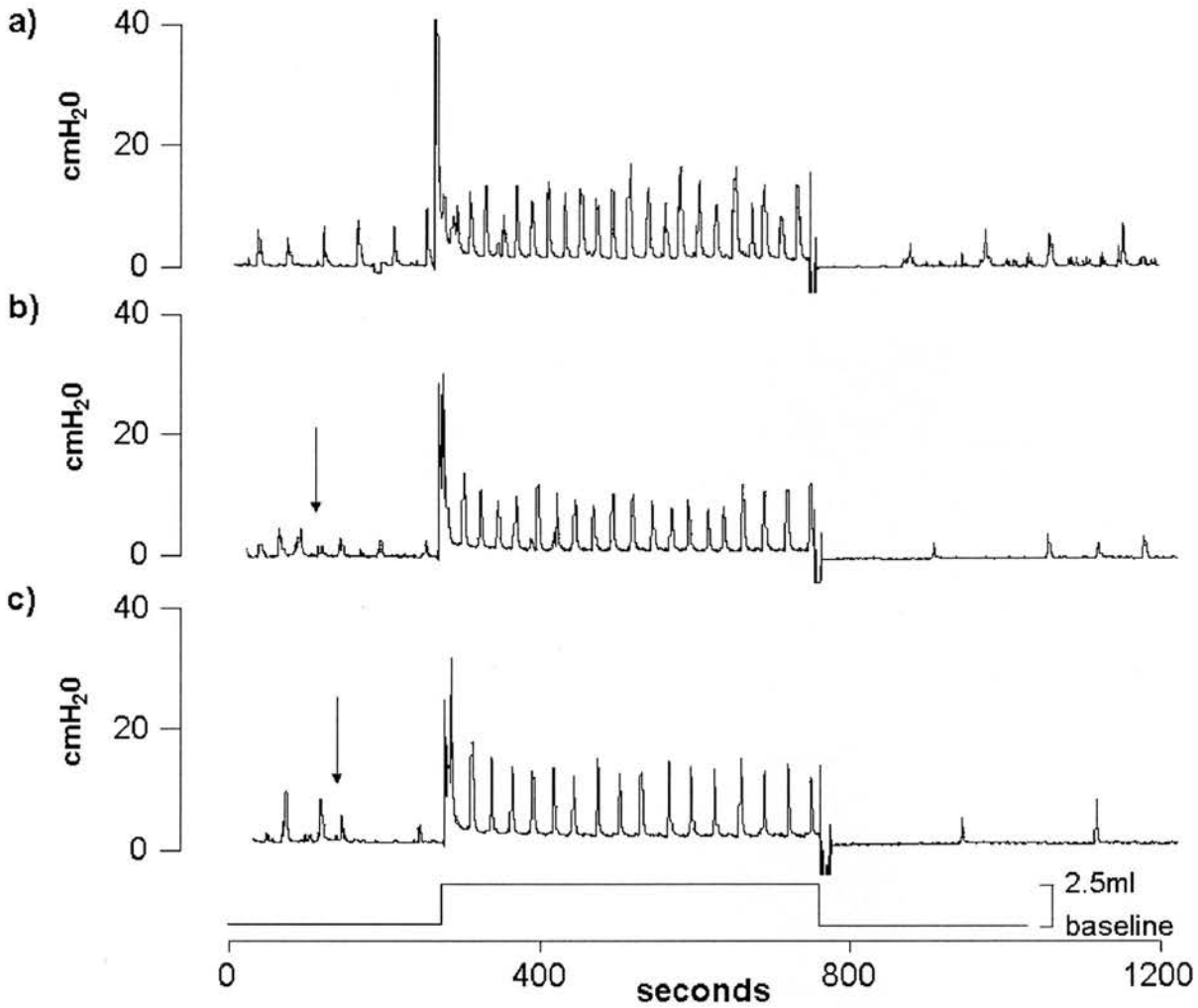


Figure 42: Typical traces showing the effects of 3 and 10 μ M pancuronium upon the volume response in the isolated guinea pig bladder; taken from the same organ preparation. Volume responses were performed in the presence of 100nM arecaidine, with a volume increase of 2.5ml lasting for 500 seconds. a) control response. b) and c) illustrate responses in the presence of 3 and 10 μ M pancuronium respectively. Arrows indicate the point at which pancuronium was added to the organ preparation. Ordinates represent pressure (cmH₂O) and the abscissa time (seconds).

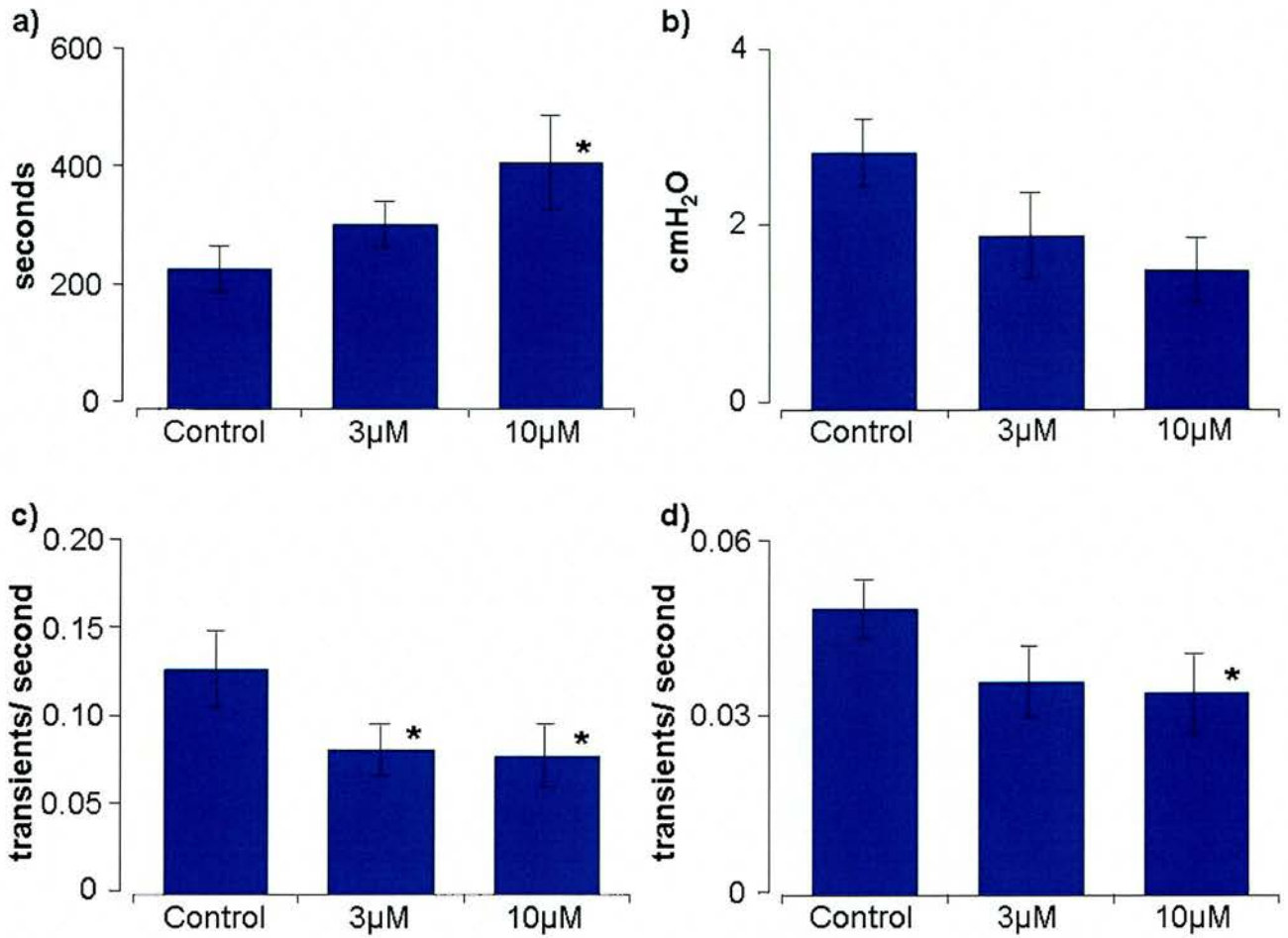


Figure 43: Data collected from 6 bladders illustrating the effects of 3 and 10 μM pancuronium on the volume response. a) illustrates the effects on the tau value of the inhibitory phase. Ordinate, tau value (seconds). b) represents the change in underlying pressure (P_{shift}). Ordinate, pressure (cmH_2O). c) and d) illustrate the effects upon frequency following volume increase (IF_{init}) and at steady state (F_{ss}) respectively. Ordinates, frequency (transients/ second). The abscissa in all panels represents the dose of pancuronium added to the organ preparation (μM). Data are shown as mean values \pm 1 SEM ($n=6$). A significant difference between doses compared to control is indicated (*), ($p<0.05$, one way ANOVA and post hoc Tukey's).

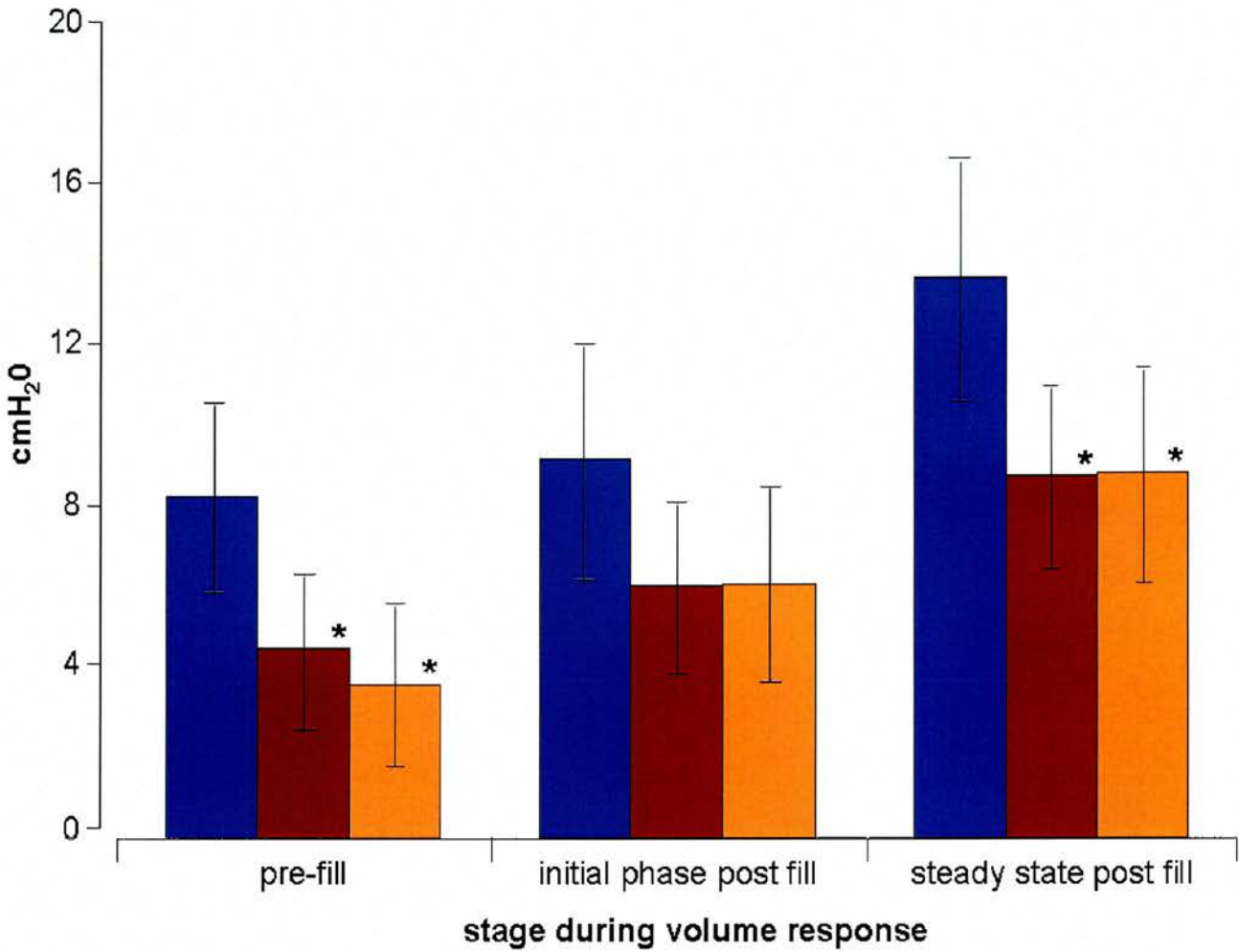


Figure 44: Data from 6 bladders showing the effects of 3 and 10µM pancuronium upon the amplitude of transient contractions during the pre-fill, initial and steady state phases. Blue column represents the control response for each stage where no pancuronium was added. Maroon, 3µM pancuronium; gold, 10µM pancuronium. Mean amplitudes are expressed ± 1 standard error of the mean, (n=6). Ordinate, amplitude (cmH₂O) and abscissa, stages of the volume response. A significant difference between doses compared to control is indicated (*), (p<0.05, one way ANOVA and post hoc Tukey's).

3.7.3 Effects of hexamethonium and pancuronium on underlying augmented autonomous activity

The effects of hexamethonium and pancuronium on underlying augmented activity, i.e. during the pre-fill phase of the volume response, are considered further. From the traces shown in Figure 45, representing typical examples of the responses following addition of hexamethonium (column A) and pancuronium (column B), no obvious effect is seen with hexamethonium. However, following addition of pancuronium to the bathing solution, at 3 and 10 μ M, a reduction in frequency and amplitude of transient contractions can be seen. In addition, at 10 μ M there seems to be a reduction in the underlying pressure.

Figure 46 summarises the mean data collected following the addition of hexamethonium and pancuronium to five and six bladders respectively. Again, no effect is seen on underlying autonomous activity following the addition of both 3 and 10 μ M hexamethonium. However, significant reductions are seen with pancuronium in the instantaneous frequency of the transients and in their amplitude at both doses, figures 40 B a) and b) respectively. Figure 46 B c) illustrates a significant reduction in underlying pressure following the addition of 10 μ M pancuronium.

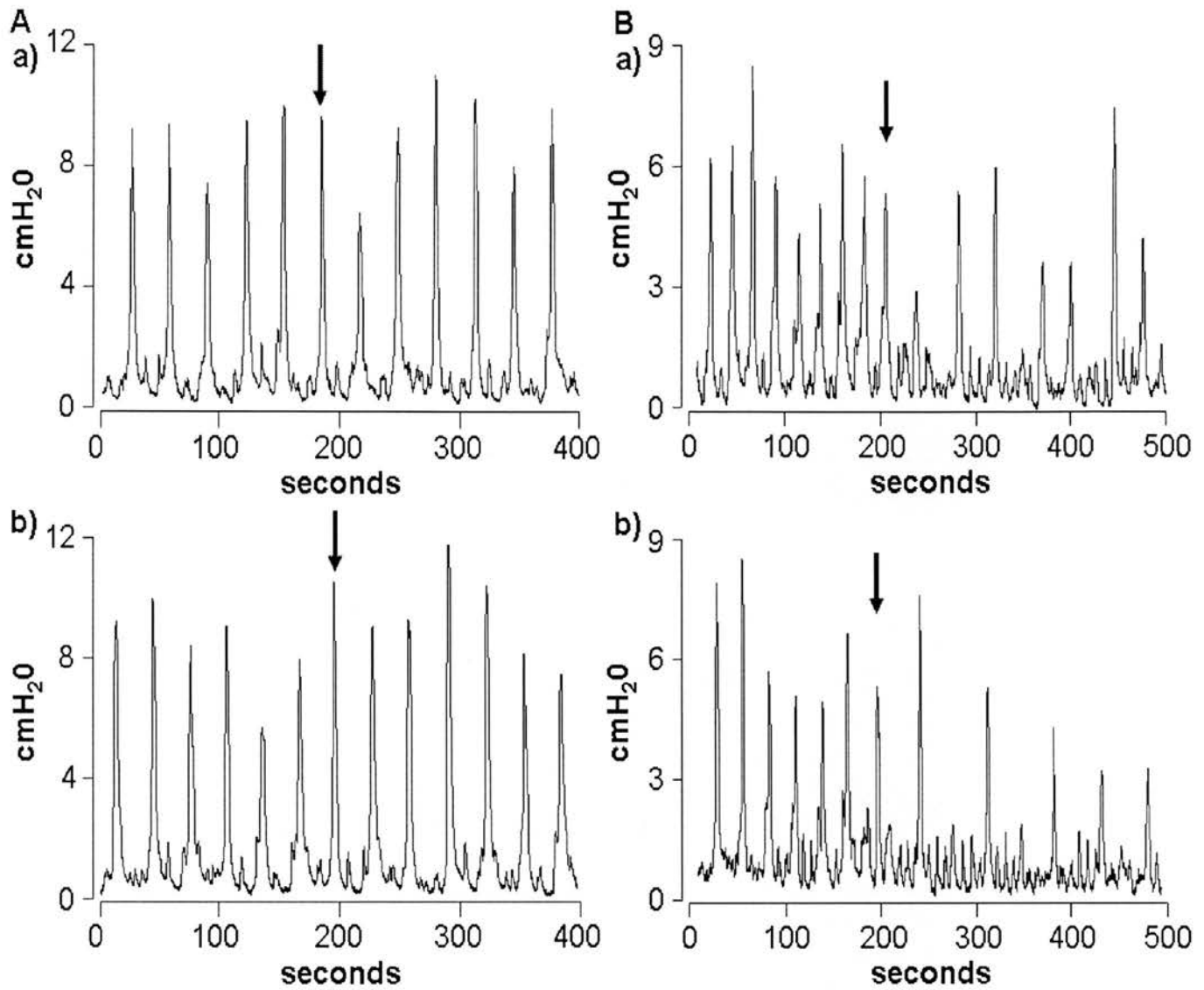


Figure 45: Typical examples of traces showing the effects of hexamethonium and pancuronium on the spontaneous activity augmented by 100nM arecaidine seen during the pre-fill phase of the volume response. Columns A and B represent the responses to hexamethonium and pancuronium respectively. Rows a) and b) represent the doses 3 and 10 μ M respectively. Arrows represent points at which drugs were added to the bathing solution of the organ preparation. In all panels ordinates represent pressure (cmH₂O) and abscissae time (seconds).

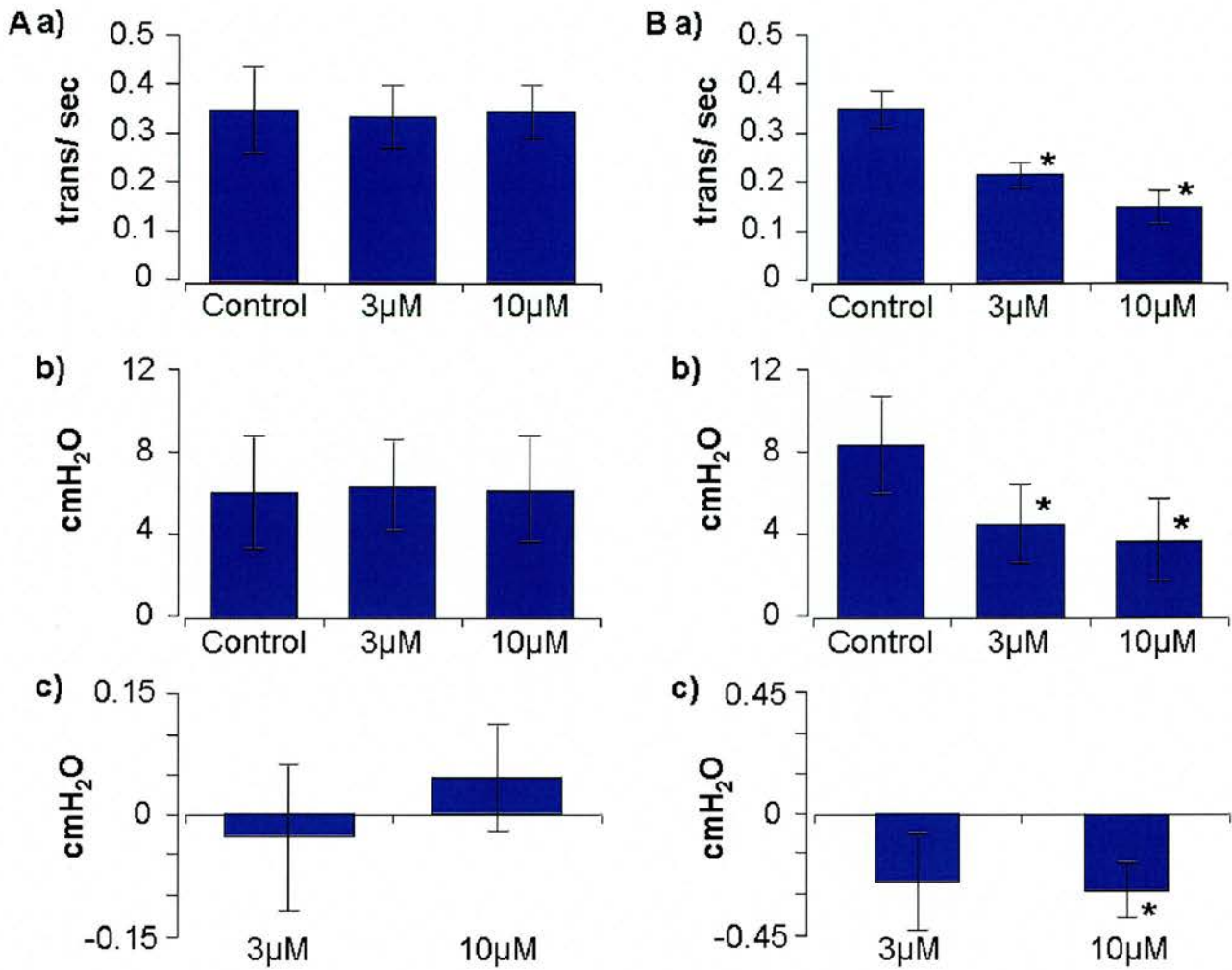


Figure 46: Data collected depicting the effects of hexamethonium and pancuronium on autonomous activity during the pre-fill phase of the volume response, columns A and B respectively. Panels a) represent instantaneous frequency of transients. b) the effects upon amplitude of transients and c) shift in underlying pressure compared to control. Mean data is expressed \pm 1 standard error of the mean. (n=5 for hexamethonium) and (n=6 for pancuronium). A significant difference between doses compared to control is indicated (*), ($p < 0.05$, a-b) one way ANOVA, c) two tailed Students t-test). Ordinates in a) represent instantaneous frequency of transients (transients/ second) and in b) – c) pressure (cmH₂O). Abscissae for all panels dose (μ M).

3.8 Effects of α/β methylene ATP on the volume response

The effects of α/β methylene ATP on the volume response in the isolated guinea pig bladder were assessed at two doses, 100 and 300nM. Both doses had similar effects by increasing frequency during the initial phase and at steady state, in addition to markedly prolonging the inhibitory phase. Figure 47 illustrates a typical example of volume responses performed on the same organ preparation in the presence of 100 and 300nM ATP. The volume change for each was 2.5ml with the increase lasting for a period of 500 seconds. These volume responses were performed in the presence of 100nM arecaidine added to the bathing solution. Figure 48 represents the traces illustrated in figures 41 a) and c) with the time scale expanded, highlighting the increase in initial frequency and the frequency at steady state between control and 300nM α/β methylene ATP.

Figure 49 summarises the mean data \pm 1 standard error of the mean from 5 isolated bladders on the effects of 100 and 300nM α/β methylene ATP on the volume response. Figure 49a) illustrates the increase in the inhibitory phase associated with α/β methylene ATP. The mean control tau value in the absence of α/β methylene ATP was 161 ± 19 seconds. In the presence of 100nM α/β methylene ATP the mean tau was 352 ± 10 seconds, which was significantly increased compared to control, ($p=0.001$). For 300nM, mean tau was 421 ± 40 seconds, again increased compared to control ($p=0.006$). Figure 49b) demonstrates that α/β methylene ATP does not significantly alter the increase in underlying pressure from baseline pre-fill levels to that at steady state, (P_{shift}). Mean P_{shift} for control, 100 and 300nM α/β methylene ATP are 2.2 ± 0.5 cmH₂O, 2.3 ± 0.8 cmH₂O and 1.3 ± 0.4 respectively. Frequencies during the initial phase were significantly increased by the two doses of α/β methylene ATP. However,

the increase in frequency seen at steady state was not significant. Figure 49c) illustrates the effects upon frequency following volume increase (IF_{init}). Mean IF_{init} for control, 100 and 300nM are 0.104 ± 0.02 transients/ second, 0.199 ± 0.02 ($p=0.013$ compared to control) and 0.24 ± 0.01 ($p=0.006$) respectively. The effects upon frequency at steady state are demonstrated in Figure 49d). Mean F_{ss} for control is 0.052 ± 0.006 transients/ second. In the presence of 100nM and 300nM α/β methylene ATP although an increase is seen these are not significant, means 0.081 ± 0.008 transients/ second and 0.084 ± 0.013 respectively.

Figure 50 shows the effects of the two doses of α/β methylene ATP upon the mean amplitude of transient contractions during the pre-fill, initial and steady state phases. It illustrates that, compared to control there is a significant reduction in the amplitude of transients in the presence of both doses during the pre-fill and initial phase. However, no significant change can be seen during the steady state phase.

The data demonstrated during these experiments reveal that the frequency of transient contractions immediately following an increase in intravesical volume is increased following the addition of α/β methylene ATP. This would suggest that purinergic receptors are involved in the regulation of frequency during this period. The fact that the frequency at steady state is not affected may suggest either a degree of desensitisation of purinergic receptors or no involvement at all. The increase seen in the inhibitory phase would suggest that purinergic receptors, to a degree, are involved in the generation or modulation of the inhibitory phase.

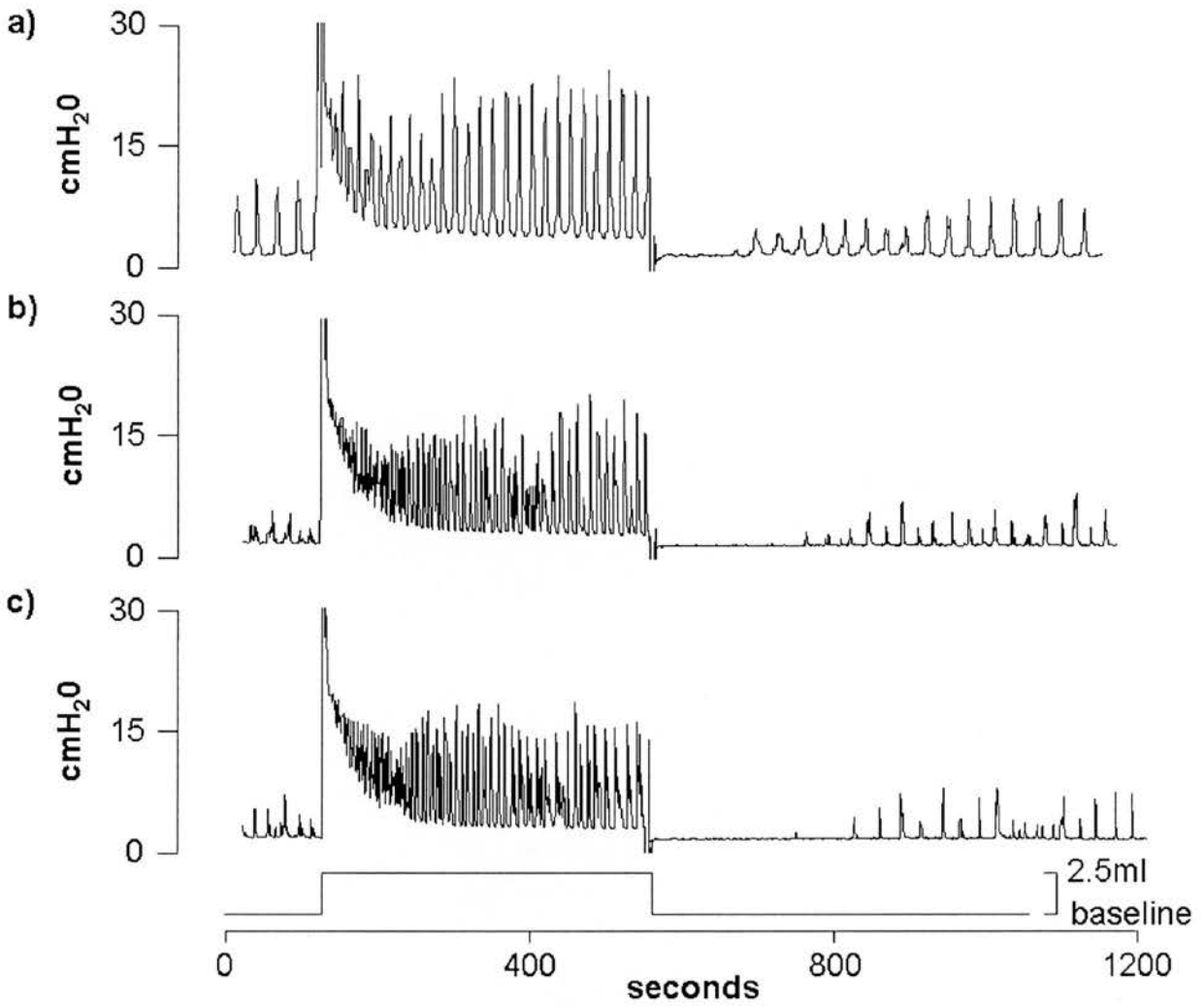


Figure 47: Typical traces showing the effects of 100 and 300nM α/β methylene ATP upon the volume response in the isolated guinea pig bladder; all traces are taken from the same organ preparation. Volume responses were performed in the presence of 100nM arecaidine, with a volume increase of 2.5ml lasting for 500 seconds. a) control response, no α/β methylene ATP added. b) and c) illustrate responses in the presence of 100 and 300nM α/β methylene ATP respectively. Ordinates represent pressure (cmH₂O) and the abscissa time (seconds).

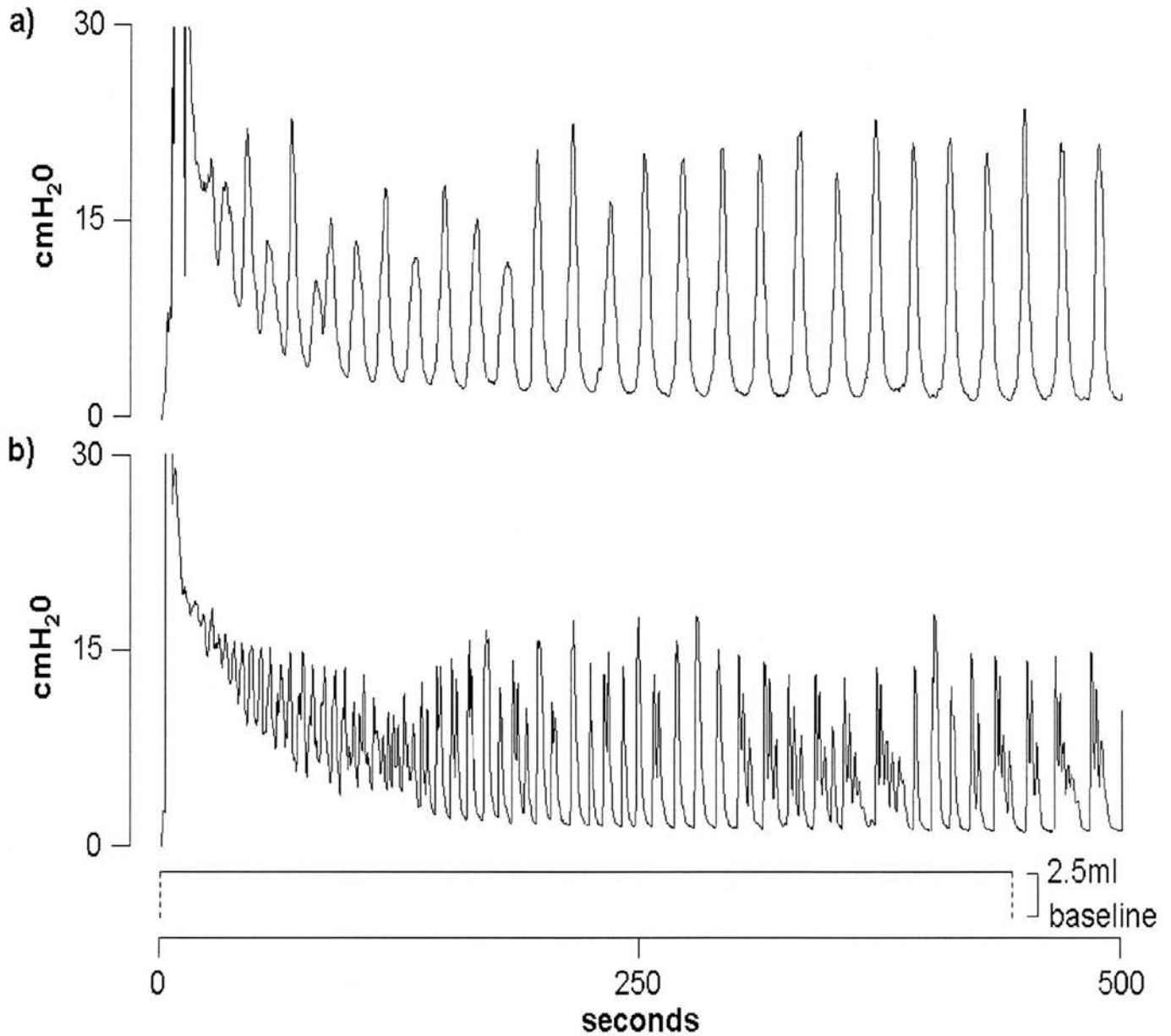


Figure 48: Traces a) and b) represent expanded versions of the traces seen in Figure 47 a) and c) respectively. a) control response, b) in the presence of 300nm. Time scale has been expanded from the point of volume increase for its 500 second duration. Ordinates, pressure (cmH₂O) and the abscissa, time (seconds). Note the increase in frequency clearly visible during the initial and steady state phases. The amount by which the intravesical volume is increased is demonstrated below the traces.

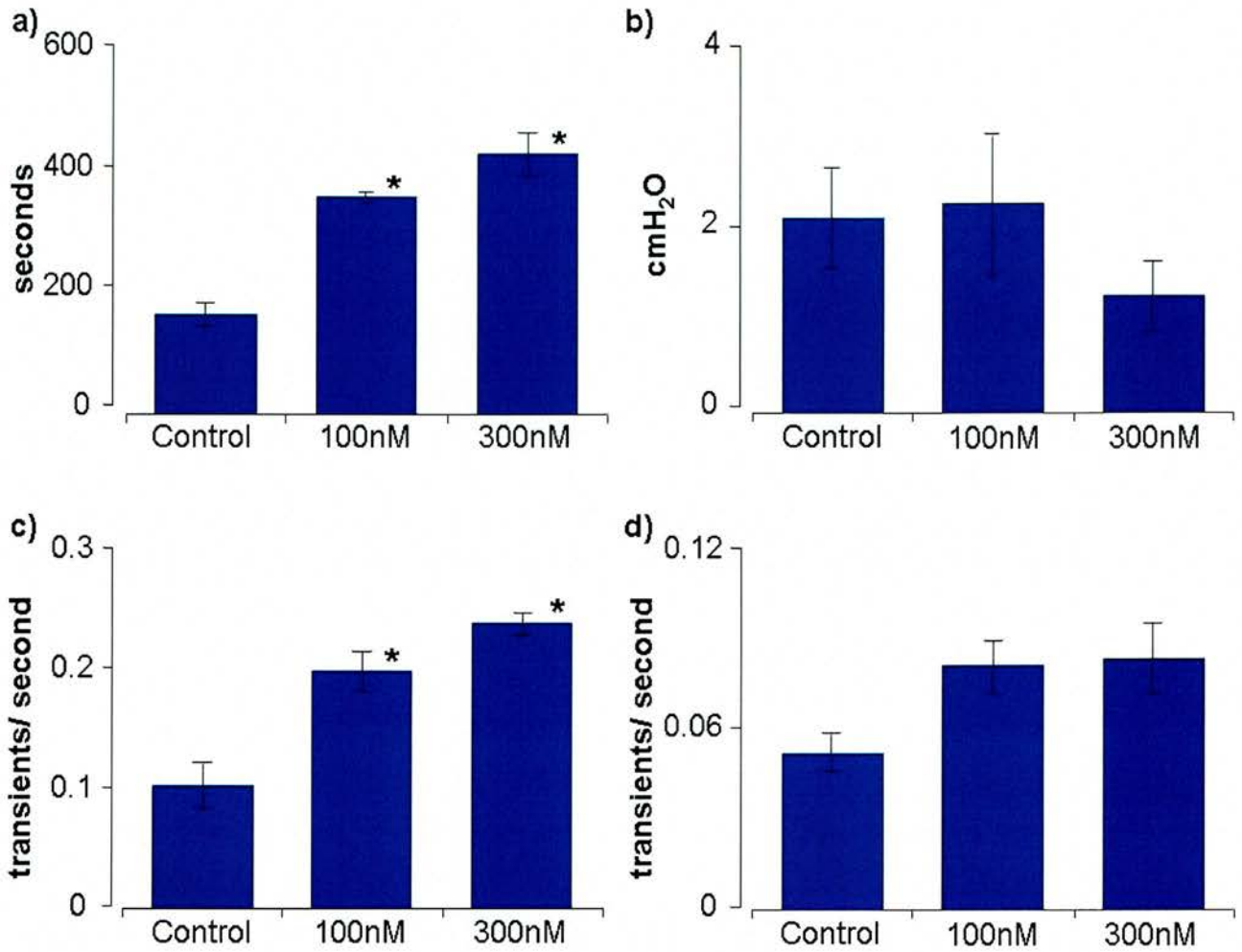


Figure 49: Data collected from 5 bladders illustrating the effects of 100 and 300nM α/β methylene ATP on the volume response. a) illustrates the effects on the tau value of the inhibitory phase. Ordinate, tau value (seconds). b) represents the change in underlying pressure (P_{shift}). Ordinate, pressure (cmH₂O). c) and d) illustrate the effects upon frequency following volume increase (IF_{init}) and at steady state (F_{ss}) respectively. Ordinates for c) and d), frequency (transients/ second). The abscissa in all panels represents the dose of α/β methylene ATP added to the organ preparation (nM). Data are shown as mean values \pm 1 SEM (n=5). A significant difference between doses compared to control is indicated (*), ($p < 0.05$, one ANOVA and post hoc Tukey's).

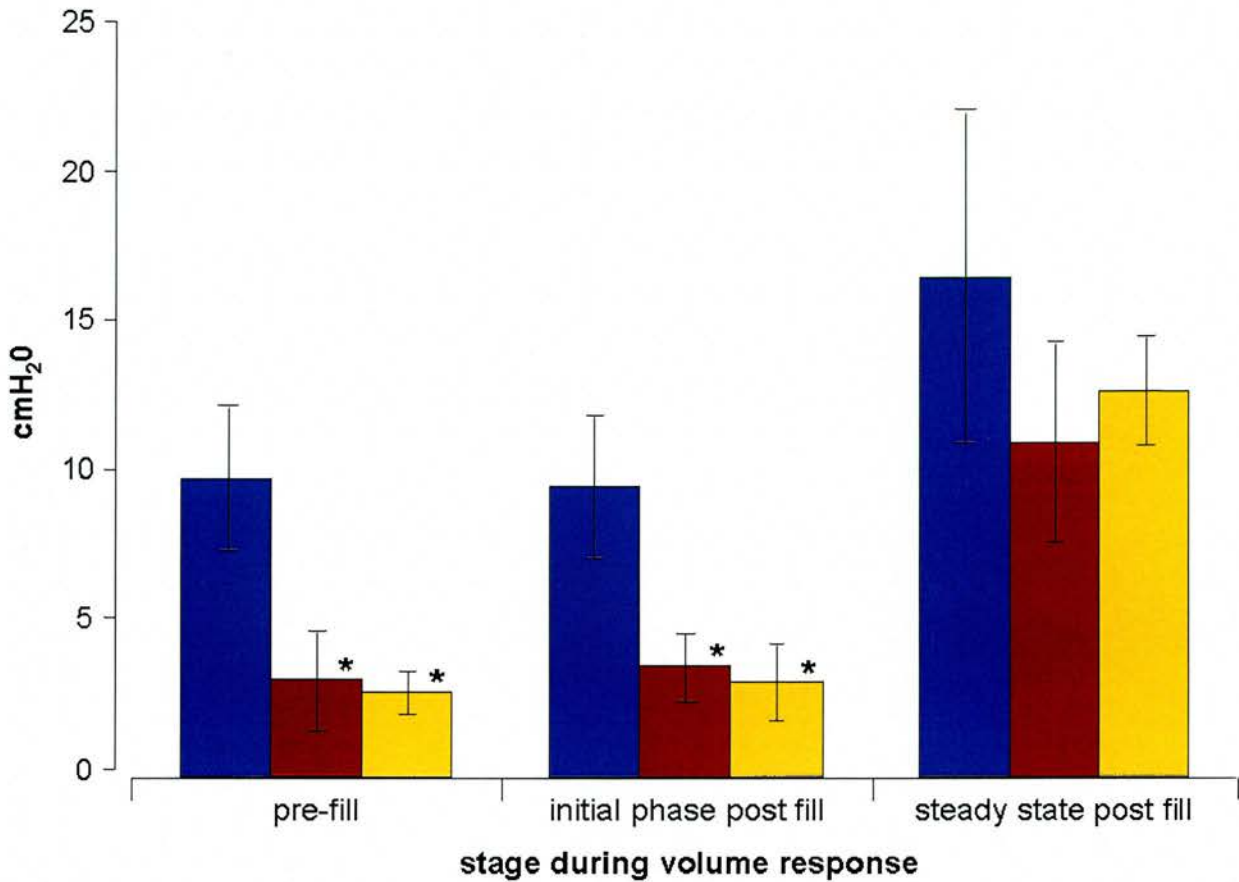


Figure 50: Data from 5 bladders showing the effects of 100 and 300nM α/β methylene ATP upon the amplitude of transient contractions during the pre-fill, initial and steady state phases. Blue column represents the control response for each stage. Maroon, 100nM α/β methylene ATP; gold, 300nM α/β methylene ATP. Mean amplitudes are expressed ± 1 standard error of the mean, (n=5). Ordinate, amplitude (cmH₂O) and abscissa, stages of the volume response. A significant difference between doses compared to control is indicated (*), ($p < 0.05$, one way ANOVA). The marked reduction in amplitude noted is probably related to the ‘aliasing’ phenomenon whereby transient contractions are precluded from achieving a full peak or trough by the onset of successive contractions.

3.9 Effects of the purinergic receptor antagonists NF 279, MRS 2179 and PPADS on the volume response

The effects of the purinergic receptor antagonists NF 279, MRS 2179 and PPADS upon the volume response in the isolated guinea pig bladder were assessed at the dose 100 μ M. Varying effects on each of the stages of the volume response were seen for each of the antagonists. NF 279, a selective antagonist of the P2X₁ receptor, was found to significantly shorten the inhibitory phase and reduce spontaneous frequency immediately after application to the isolated bladder. It also served to increase frequency immediately following a volume increase (IF_{max}). No effect was seen upon frequency at the steady state (F_{ss}). MRS, a selective P2Y₁ antagonist, had no significant effect on either the inhibitory phase or frequency at steady state (F_{ss}). Though it did significantly reduce the frequency of spontaneous activity following its application and increase the spontaneous frequency immediately following volume increase (IF_{max}). Data for these two antagonists were collected from the same guinea pig preparations and as such their control responses were the same. The mean data \pm 1 standard error of the mean for NF and MRS are shown in table 9.

The effects of PPADS, a non selective P2 receptor antagonist, were more pronounced. A marked reduction in the inhibitory phase was seen with a significant increase seen in spontaneous activity following application, frequency following volume increase (IF_{max}) and the frequency at the steady state (F_{ss}). Since this data was acquired from a separate set of bladder preparations results are shown in table 10. Typical traces showing the responses of these three antagonists on volume induced activity are shown in Figure 51. Figure 52 illustrates the effects of the antagonists upon the four parameters; inhibitory phase, frequency following addition of antagonist to the

preparation, frequency following volume increase (IF_{max}) and frequency at steady state (F_{ss}).

	Inhibitory phase (secs)	Frequency pre-vol. increase (Hz)	IF_{max} (Hz)	F_{ss} (Hz)
Control	142 ± 4	0.039 ± 0.001	0.14 ± 0.01	0.061 ± 0.0006
NF 279	$108 \pm 3^*$ (0.007)	$0.034 \pm 0.002^*$ (0.03)	$0.16 \pm 0.004^*$ (0.03)	0.058 ± 0.003 (0.165)
MRS 2179	164 ± 15 (0.07)	$0.037 \pm 0.001^*$ (0.007)	$0.179 \pm 0.011^*$ (0.0004)	0.069 ± 0.006 (0.165)

Table 9: Data illustrating the effects of the purinergic antagonists, NF 279 and MRS 2179, upon the inhibitory phase, frequency of spontaneous activity immediately after application of the agonist to the bladder preparation, frequency immediately following volume increase (IF_{max}) and the frequency at steady state (F_{ss}). Mean values for 4 separate guinea pig bladder preparations are shown ± 1 standard error of the mean (mean ± 1 SEM). Significant differences compared to control values are highlighted by an asterisk ($p<0.05$), with the respective p – value quoted (two tailed students t-test).

	Inhibitory phase (secs)	Frequency pre-vol. increase (Hz)	IF_{max} (Hz)	F_{ss} (Hz)
Control	153 ± 10	0.036 ± 0.002	0.125 ± 0.003	0.054 ± 0.003
PPADS	$62 \pm 1^*$ (0.002)	$0.055 \pm 0.003^*$ (0.0002)	$0.16 \pm 0.007^*$ (0.02)	$0.096 \pm 0.004^*$ (0.012)

Table 10: Data illustrating the effects of the purinergic antagonist, PPADS, upon the inhibitory phase, frequency of spontaneous activity immediately after application of the agonist to the bladder preparation, frequency immediately following volume increase (IF_{max}) and the frequency at steady state (F_{ss}). Mean values for 4 separate guinea pig bladder preparations are shown ± 1 standard error of the mean (mean ± 1 SEM). Significant differences compared to control values are highlighted by an asterisk ($p<0.05$), with the respective p – value quoted (two tailed students t-test).

The purinergic antagonists NF 279 and PPADS both reduce the inhibitory phase suggesting a role for purinergic receptors in either the generation or prolongation of the inhibitory phase. The action of NF 279 suggests a specific role for $P2X_1$ receptors in

this activity, however, since no effect was seen with MRS 2179 P2Y₁ receptors are unlikely to be functionally relevant. With regards to underlying spontaneous activity both NF 279 and MRS 2179 significantly reduce the frequency of contractions suggesting that P2X₁ and P2Y₁ receptors have a positive effect in the modulation of frequency. The non-specific effect of PPADS suggests however, that the overall purinergic effect upon the modulation of frequency is inhibitory, (although this contradicts the results of α/β methylene ATP possible reasons are discussed later). Furthermore, following an increase in volume the immediate frequency is increased by the three purinergic antagonists suggesting that purinergic activity during this time period serves to inhibit frequency. Although the effects of NF 279 and MRS 2179 upon frequency at steady state suggest little modulation by P2Y₁ and P2X₁ receptors the effects of PPADS again suggest an overall inhibitory role for purinergic receptors in the modulation of spontaneous transient contractions once a steady state has been achieved.

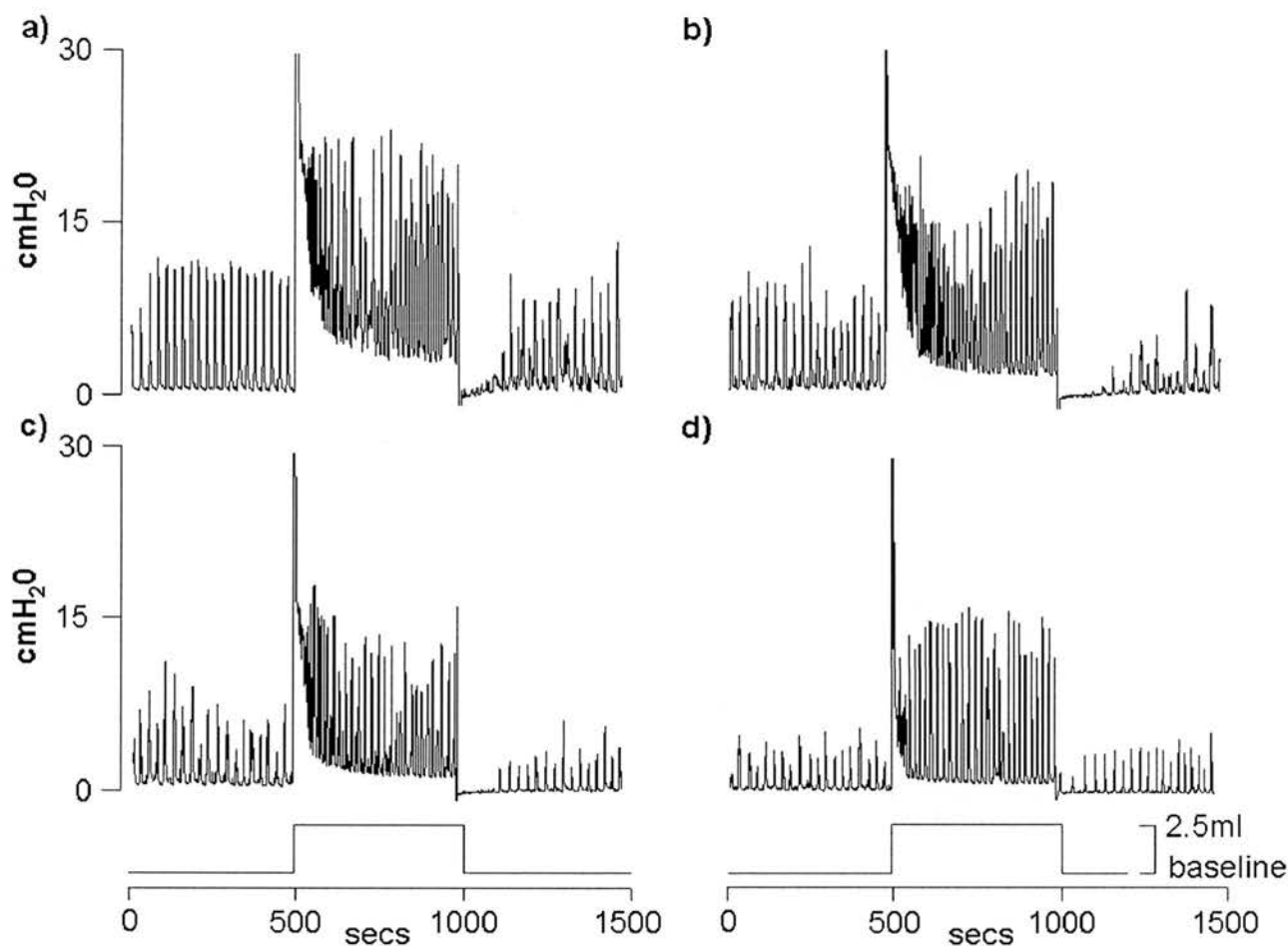


Figure 51: Typical traces illustrating the effects of the purinergic antagonists upon the volume response in the isolated guinea pig bladder; traces a, b, c are taken from the same organ preparation, d is taken from a separate preparation. Volume responses were performed in the presence of 100nM arecaidine, with a volume increase of 2.5ml lasting for 500 seconds illustrated at the base of the figures. The points at which these agents were added to the organ preparation are not shown. a) control response. b) 100µM MRS 2179. c) 100µM NF 279. d) 100µM PPADS. Ordinates represent pressure (cmH₂O) and the abscissa time (seconds).

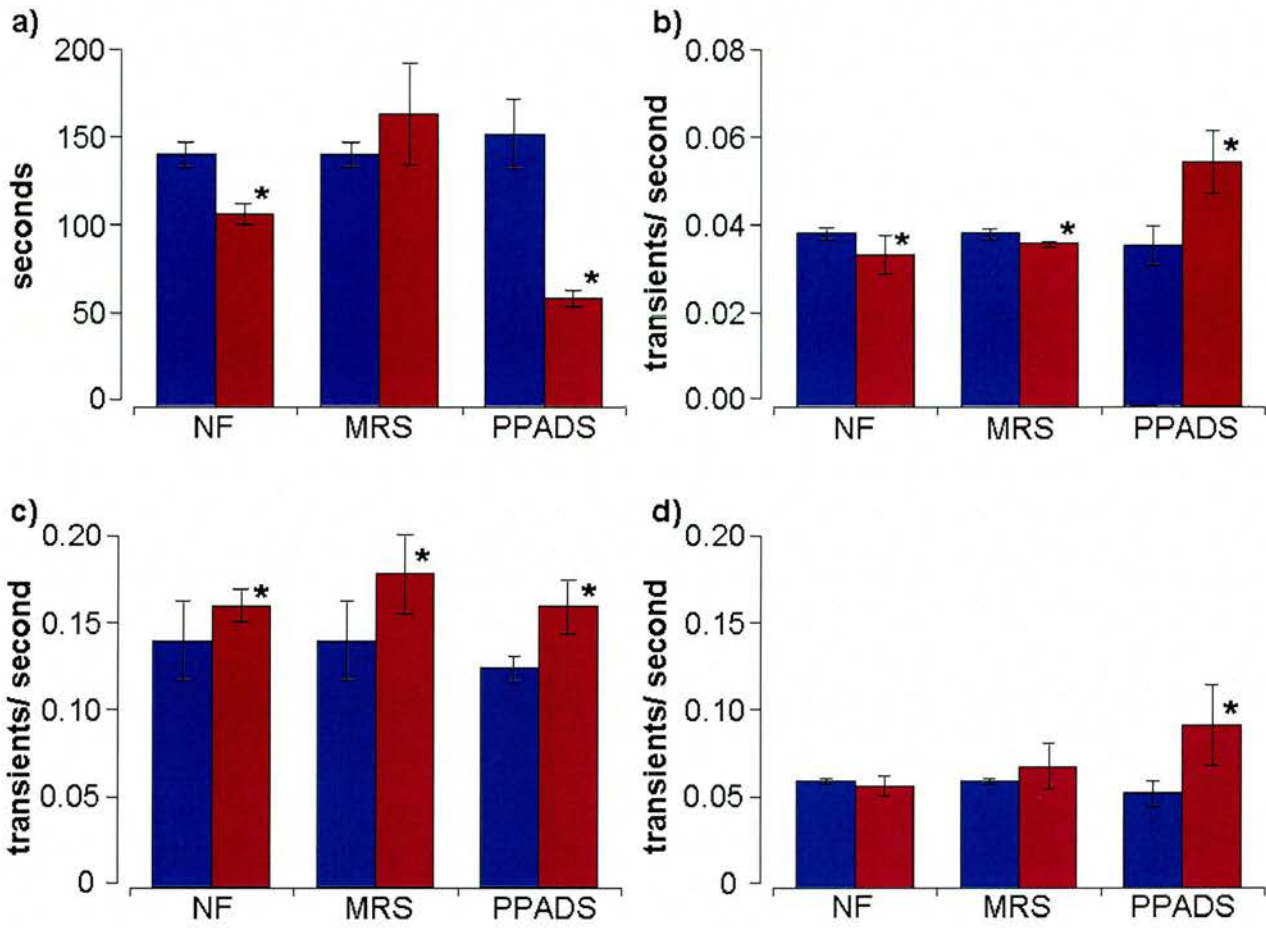


Figure 52: Data collected from 4 bladders illustrating the effects of 100μM NF 279, MRS 2179 and PPADS on the volume response. a) illustrates the effects on the inhibitory phase. Ordinate, tau value (seconds). b) represents the change in spontaneous frequency following addition of antagonist to the preparation. Ordinate, transients/second c) and d) illustrate the effects upon frequency following volume increase (IF_{init}) and at steady state (F_{ss}) respectively. Ordinates, frequency (transients/ second). The abscissa in all panels represents the antagonist added to the organ preparation. Blue represent the control value and maroon represents the value with antagonist added. Data are shown as mean values \pm 1 SEM (n=4). A significant difference between antagonist compared to control is indicated (*), ($p < 0.05$, Student t-test).

DISCUSSION

Chapter Four

4.1 Introduction

During the course of these experiments the isolated guinea pig bladder was found to demonstrate a number of complex responses that would suggest the presence of a number of interrelated mechanisms responsible for both the generation and co-ordination of spontaneous activity. Following the addition of the muscarinic agonists, arecaidine and carbachol, two distinct phases could be seen; an 'initial phase' whereby underlying pressure and frequency of transient contractions increase followed by a 'steady state' whereby frequency and basal pressure reduces down to steady, regular levels. Carbachol was found to significantly increase the frequency of contractions during the initial phase, whereas arecaidine did not. This in conjunction with the effects of 4-DAMP (M_3 antagonist) in reducing frequency and AF-DX (M_2 antagonist) having little effect, suggest that the mechanisms regulating frequency during the initial phase are sensitive to cholinergic, and more specifically, M_3 mediation. A similar response is seen following an increase in intravesical volume, again consisting of an initial phase and a steady state. However, following a reduction in intravesical volume (back to baseline volumes) a period of quiescence is seen followed by a gradual return of spontaneous activity back to the levels seen pre volume change. The inhibitory phase was found to increase with increased amounts of volume increase/ decrease in addition to longer durations of volume increase. Arecaidine, hexamethonium, pancuronium and α/β methylene ATP also affected the inhibitory phase suggesting that the mechanisms involved in the generation and modulation of this quiescence are regulated to a degree by muscarinic, nicotinic and purinergic factors.

With data emerging to question our basic understanding of the bladder it has become apparent that our traditional views of its function, structure and pathology need to be readdressed. New data has demonstrated complex structures within the bladder wall that potentially could account for the novel mechanisms that have been recently documented. Additionally, in retrospect review of older more established data also suggests that these traditional views cannot be fully supported. In this regard, review of clinical cystometric data over the years, albeit sparse, fails to support the idea that antimuscarinic medications exert their beneficial actions through the inhibition of detrusor contraction alone, overactive or otherwise. They do, however, demonstrate an improvement in sensory parameters (Finney et al, 2006) and in doing so question the role of acetylcholine (ACh) acting solely as a mediator of global detrusor contraction. However, it must be acknowledged that at doses higher than those used in routine clinical practice antimuscarinics are capable of reducing contractility; which previously supported the traditional view. In context therefore, these observations go towards suggesting the presence of two cholinergically mediated mechanisms within the bladder. One relatively sensitive to cholinergic excitation involved in the generation or mediation of sensation and another, less sensitive one, responsible for detrusor contraction.

In attempting to account for these observations an alternative view of bladder regulation needs to be considered taking into account recent data. The bladder has been shown to be capable of spontaneous rhythmical contractions associated with bursts of afferent activity (Andersson and Yoshida, 2003; Vaughan and Satchell, 1995); suggesting the presence of cells within the bladder capable of spontaneous pacemaker activity in addition to the presence of rapidly responding stretch receptors.

Furthermore, within both the mouse and guinea pig, this spontaneous activity (termed 'Autonomous activity') has been found to be modulated in a manner suggesting the presence of local reflexes within the bladder wall (Drake et al, 2003). A major component of these local reflexes is the positive relation between intravesical volume and the frequency/ amplitude of transient contractions. With strong evidence to support that afferent discharge is related to these contractions (Andersson and Yoshida, 2003; Vaughan and Satchell, 1995) it can be speculated that 'Autonomous activity' has a role in the generation +/- mediation of sensation and therefore, the perception of bladder volume. Since 'Autonomous activity' has a degree of cholinergic modulation, with increases in activity associated with increasing doses of muscarinic agonist (Drake et al, 2003), there is further support for a sensory mechanism with cholinergic components. Therefore, 'Autonomous activity' may represent the relatively sensitive mechanism described previously, upon which antimuscarinics act and through which they exert their beneficial effects.

Evidence for the presence of 'Autonomous activity' has been seen both *in vitro* and *in vivo* during animal studies (Streng et al, 2006). In the human, activity comparable to 'Autonomous activity' can be seen upon ambulatory urodynamic monitoring. However, it is only recently, with the advent of modern technology, that mechanisms and structures capable of co-ordinating these activities have been identified within the bladder wall. Cells with 'pacemaker' function analogous to the Interstitial Cells of Cajal, found in the gut, have been identified lying in close proximity to the urothelium with a further set seen in and around the detrusor muscle. Ganglia have also been identified, with some receiving projections from the urothelium. The role of these ganglia is uncertain though they would seem to be of some significance since the

urothelium has been found to release adenosine triphosphate (ATP), nitric oxide (NO) and acetylcholine (ACh) in response to stretch. Therefore, these structures have the capacity to account for the generation of spontaneous activity seen in 'Autonomous activity', in addition to modulation and co-ordination of global contraction leading to changes in its frequency and amplitude, potentially through a number of integrating excitatory and inhibitory inputs.

Previous work has been performed upon the response of 'Autonomous activity' to certain physiological stimuli in an attempt to relate structure to function. This work has mostly been performed upon the isolated bladder under iso-volumetric conditions. However, as the bladder undergoes both filling and emptying further complex responses are seen (Lagou et al, 2004), suggestive of a dynamic process whereby the mechanisms behind 'autonomous activity' are potentially seen throughout all stages of the micturition cycle. In an attempt to categorise these mechanisms further the effects of muscarinic stimulation/ inhibition, ATP and nicotinic antagonists upon the volume response (Lagou et al, 2004) were assessed. Each group of experiments are discussed individually within their own context, followed by a broader discussion including further potential clinical ramifications.

4.2 The effects of the muscarinic agonists, arecaidine and carbachol, and the muscarinic antagonists, 4-DAMP and AF-DX, upon agonist induced phasic activity under iso-volumetric conditions

It is widely accepted that antimuscarinic drugs are effective in reducing the symptoms of OAB. Contraction of the smooth muscle cells in the bladder involves predominantly activation of M_3 muscarinic receptors (Andersson and Arner, 2004). However, it is unlikely that it is this population of M_3 receptors that are involved in the therapeutic action of antimuscarinic drugs (Andersson and Yoshida, 2003; Finney et al, 2006; Andersson, 2002; Kim et al, 2005; Yokoyama et al, 2005). In addition, it is emerging that the antimuscarinic drugs diminish sensations of urge and so reduce the need for frequent episodes of micturition. Thus, the therapeutic site of action of the muscarinic drugs must be on M_3 receptors operating within a system involved in the generation or modulation of sensations during the filling phase of the micturition cycle. However, the location of these receptors is not known nor is their role in the generation or modulation of sensation.

A further complication concerns the location and function of M_2 receptors in the bladder wall. There are data available reporting that there are more M_2 receptors in the bladder than M_3 . Despite this, their exact location or the physiological roles they serve is unknown (Barlow and Shepherd, 1986; Hegde et al, 1997; Yamanishi et al, 2001; Yamanishi et al, 2002; Braverman et al, 2002; Chess-Williams, 2004). Possible mechanisms involving the M_2 dependent regulation of detrusor contraction have been suggested. M_2 activation inhibits adenylyl cyclase resulting in a decrease in intracellular cAMP. Since a decrease in cAMP leads to an increase in contractility this was proposed as a possible function of M_2 receptors in the regulation of bladder contractility. In

addition, M₂ receptors have been demonstrated within the urothelial layer with some studies to suggest a prevalence of M₂ receptors within the urothelium (Hawthorn et al, 2000). Though more recent studies using real time quantitative polymerase chain reaction (RT-QPCR) have suggested the presence of functional M₂ receptors but dispute the prevalence of the density in the urothelium over that of the detrusor (Tyagi et al, 2006). However, in any case the presence of M₂ receptors within the detrusor suggests that the urothelium, to a degree, might be involved in the regulation of detrusor contractility.

It has been recognised for more than 120 years that the bladder is not quiescent during the filling phase. Small transient rises in pressure occur that become larger and more frequent as the bladder fills (Mosso and Pellacani, 1882; Sherrington C, 1892; Vaughan and Satchell, 1995; Streng et al, 2006). It was proposed that this non-micturition activity generates afferent discharges that contribute to bladder sensation (Starling E, 1905; Gillespie J, 2005). Phasic activity similar to the non-micturition activity seen *in vivo* can be detected in the isolated bladder (Gillespie J, 2005; Sherrington C, 1892). Using this preparation it was shown that phasic activity, also known as ‘autonomous activity’ can be augmented by muscarinic agonists and this ‘augmented activity’ is increased by ATP and substance P (Gillespie J, 2005a). It can also be inhibited by calcitonin gene related peptide and noradrenaline (Gillespie J, 2005a; Gillespie J, 2004c). This has lead to the idea that the non-micturition activity can be modulated to alter the sensitivity and output of this motor/ sensory system (Gillespie J, 2005). As the non-micturition activity is influenced by muscarinic stimulation it follows that reduction of this muscarinic component by antimuscarinic drugs will reduce sensation.

Thus, this mechanism could represent the therapeutic site of action of the antimuscarinic drugs.

The observations described in these experiments illustrate the responses of the whole isolated bladder to muscarinic stimulation. Exposure to agonist results in a complex pattern of transient rises in intra-vesical pressure which change during the period of application. Immediately following exposure to agonist the frequency of transients rises rapidly to a maximum and then declines to a steady level. The value of the maximum frequency during the initial phase (F_{init}) increases with increasing agonist concentration. In contrast, the frequency of the transients in the steady state does not tend to change with different agonist concentrations. The amplitude of the transients in the initial phase is smaller and more irregular than those in the steady state. At present, the mechanisms which generate and regulate both frequency and amplitude of the phasic activity are not entirely understood. However, it is unlikely that they originate as a result of activation of the M_3 receptors situated on the detrusor muscle (Gillespie, 2005b).

It has been shown that the transient rises in pressure induced by muscarinic agonists are accompanied by propagating waves of contraction that can be visualised over large parts of the bladder wall which are accompanied by local stretches (Drake et al, 2003a; Drake et al, 2003c). This has led to the idea that the transients are initiated by a 'pacemaker' like mechanism and that the activity is then distributed throughout the bladder wall by a specialised distribution network. Structurally, this network may consist of a combination of nerves, intramural ganglia and interstitial cells (Gillespie

2004; Gillespie 2005b). The observations that the initial frequency of the transients is dependent on muscarinic agonist stimulation, suggests an involvement of muscarinic receptors on the cells directly responsible for the generation of pacemaker activity.

That the agonist dependent changes in the initial frequency are sensitive to the relatively M_3 selective muscarinic antagonist 4-DAMP (Barlow and Shepherd, 1986), further suggests that the cells performing the pacemaker function express muscarinic receptors and for these to be predominantly the M_3 receptor subtype. The relative insensitive effects of 4-DAMP upon amplitude however, suggests the mechanisms regulating the amplitude of transient contractions are not exclusively M_3 mediated; involving both M_3 and M_2 receptor subtypes.

The amplitude of the transients was related, to a degree, to the concentration of the muscarinic agonist and the degree of muscle activation resulting from the spread of activity over the bladder surface. This suggests that the propagation of the activity is regulated by a muscarinic mechanism that might be more dominated by M_2 than M_3 receptors. This is supported by the finding that the relatively M_2 specific antagonist AFDX-116 (Hammer et al, 1986) does not markedly influence the initial transient rise in frequency. However, AFDX-116 does affect the amplitude of the transients.

The rapid activity generated in the pacemaker mechanism by muscarinic stimulation is transient: the stimulus activates an initial burst of activity which declines to a steady state. As already stated, the frequency of the initial burst of activity depends on the applied agonist concentration and is inhibited by 4-DAMP. In contrast, the frequency

of the transients in the steady state is less sensitive to agonist concentration and to 4-DAMP. This could suggest that the initial and steady state transients may be dependent upon different but interrelated mechanisms. In keeping with this idea the nicotinic ligand Lobeline is capable of producing slow large transient rises in pressure similar to those seen in the steady state but does generate a rise in basal pressure or burst of high frequency activity (Drake et al, 2003c). Suggesting the presence of two mechanisms generating and propagating phasic activity in the guinea pig bladder; a pacemaker, used to determine the frequency of the phasic activity, and a distribution mechanism used to propagate activity in waves of contraction throughout the bladder. An illustration demonstrating these principles is shown in Figure 53. Since the initial burst of activity is activated by muscarinic agonists and inhibited by 4-DAMP it is likely that this pacemaker mechanism is regulated by predominantly M_3 muscarinic receptors rather than M_2 (i.e. $M_3 > M_2$). In contrast the large steady state transients are less sensitive to the M_3 antagonist and may be dependent on M_2 operating via changes in intracellular cAMP ($M_2 > M_3$). Also, there must be a component of bladder activity resulting from direct activation of the M_3 muscarinic receptors on the smooth muscle. Activation of the smooth muscle directly is likely to result in simultaneous activation of all of the muscle resulting in a contracture.

The observation that the large transients generated during the steady state of an agonist complex response are relatively insensitive to the M_3 specific antagonist 4-DAMP suggests that cholinergic stimulation of these cells may be via M_2 receptors. Especially since the specific M_2 antagonist AFDX-116 reduces the amplitude of the transients. The large transients in the steady state are larger and more regular when induced by arecaidine compared to carbachol. Arecaidine is a muscarinic agonist with

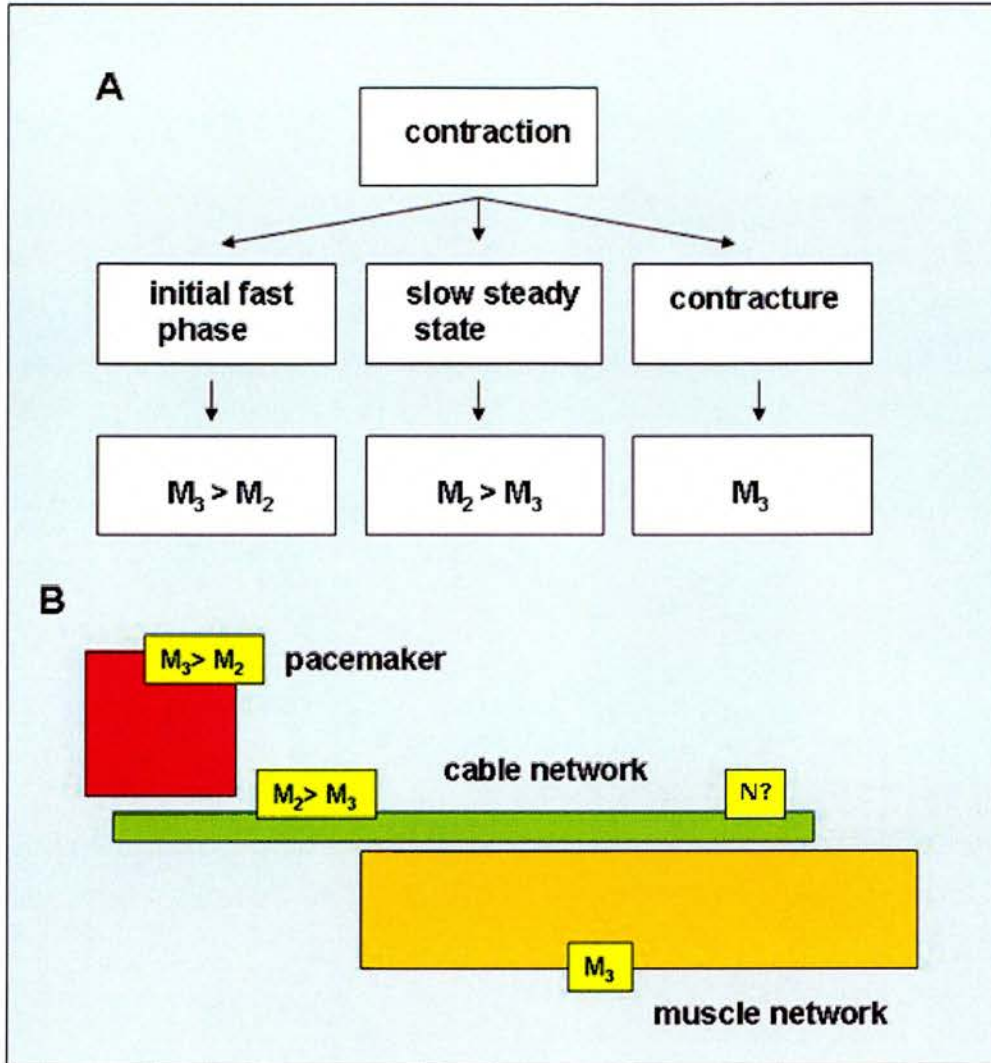


Figure 53: A schematic diagram suggesting a possible arrangement of mechanisms to account for the complex actions of carbachol and arecaidine on the isolated whole bladder preparation. A illustrates a block diagrammatic representation of the three basic phenomena. The different components shown are: the initial fast component of phasic activity, the steady state component of the phasic activity and the initial rise in basal pressure. B is a cartoon suggesting how these phenomena might be related to cellular mechanisms and anatomical features within the bladder. The possibility is put forward that there is (i) a pacemaker element, driven by a mechanism dominated by M_3 receptors. (ii) A distribution network, involving M_2 components. This network contacts and communicates with the muscle resulting in phasic contractile activity. (iii) Contractions of the detrusor, controlled by M_3 receptors possibly activated directly by efferent parasympathetic cholinergic nerves. (iv) A nicotinic receptor on the distribution network, bypassing the mechanisms involved in the ‘fast’ phase (as demonstrated by the effects of Lobeline; Drake et al, 2003c).

a reported greater selectivity for M₂ receptors over M₃ (Barlow and Weston-Smith, 1985). Thus, the cells responsible for the co-ordination of transient contractions may be activated and regulated by M₂ receptors. Activation of M₂ muscarinic receptors in many cells results in the inhibition of adenylate cyclase and a fall in intracellular cAMP (Andersson and Arner, 2004). If this is correct then activity in these cells is regulated by cAMP; a fall in cAMP results in an increase in excitability. In keeping with this idea it has been shown that procedures which elevate cAMP (forskolin and phosphodiesterase inhibitors) inhibit phasic activity in the isolated bladder preparation (Gillespie, 2004d). This outline would offer a possible explanation and role for the presence of M₂ receptors in the bladder.

There is recent evidence to support the presence of a structural framework within the bladder capable, in part, of generating and co-ordinating the activity described above. The presence of a heterogeneous network of cells, interstitial cells, has been demonstrated associated with the detrusor which has the potential to function as a coordinating network, controlling the waves of detrusor contraction (Smet et al, 1996; McCloskey and Gurney, 2002; Hashitani et al, 2004; Gillespie et al, 2005; Davidson and McCloskey, 2005; Gillespie et al, 2006; Gillespie et al, 2006a; Lagou et al, 2006). In both the guinea pig and the mouse the responsiveness of interstitial cells to nitric oxide with a resultant elevation in intracellular cGMP has been used to identify the distribution of these cells (Gillespie et al, 2005; Gillespie et al, 2006; Lagou et al, 2006). In the guinea pig three types have been identified to be associated with the outer muscle layer. These have been described by Gillespie et al, (2005), as cells running on the outer margin of the bladder wall (muscle coat interstitial cells; MC-ICs), cells on the surface of the muscle bundles (surface muscle interstitial cells; SM-ICs) and cells

within the muscle bundles (intramuscular interstitial cells; IM-ICS). Furthermore, the IM-IC's may consist of two populations of cells; those expressing choline acetyl transferase (ChAT) and those that do not, (Gillespie et al, 2006). In the mouse there appear only to be two types of interstitial cell associated with the outer muscle layers: IM-ICs and SM-ICs. In both guinea pig and mouse the SM-ICs and IM-ICs come into close contact with nerve fibres suggesting that activity in these cells might be under neural control, possibly cholinergic (Smet et al, 1996; McCloskey and Gurney, 2002; Hashitani et al, 2004; Gillespie et al, 2005; Davidson and McCloskey, 2005; Gillespie et al, 2006; Gillespie et al, 2006a; Lagou et al, 2006). Corroborating these structural findings, interstitial cells, isolated from the detrusor are activated by muscarinic agonists (McCloskey and Gurney, 2002), in addition, activity in isolated bundles of smooth muscle cells has been found to originate at specific points on the bundle surface, presumably the interstitial cells (Hashitani et al, 2004). These findings support the concept that cholinergic nerve mediated activity in the network of interstitial cells is transferred to the underlying muscle bundles resulting in coordinated, episodic waves of contraction. Further to this argument, nitric oxide has been shown to selectively reduce phasic activity in the isolated mouse bladder (Lagou et al, 2006). As the major cell type sensitive to nitric oxide in the mouse bladder are the interstitial cells, this suggests that these cells play an integral part in the generation of phasic activity.

As the pacemaker mechanism is the key component to phasic activity, inputs from various sources must integrate here to exert their effect. However, the location and nature of this potential pacemaker mechanism is not known. In the guinea pig, a recent study has demonstrated the presence of a sub-urothelial ganglionic network. The neurones of which are contacted by different types of nerve fibre. Three types have

been identified: fibres expressing calcitonin gene related peptide, fibres expressing choline acetyl transferase (ChAT) and neurofilament protein (ChAT/NF⁺) and fibres expressing ChAT but not neurofilament (ChAT/NF⁻), (Gillespie et al, 2006a). The calcitonin gene related peptide (CGRP) and ChAT/NF⁻ fibres may be collaterals of sensory fibres which appear to originate in the sub-urothelial layer. The ChAT/NF⁺ fibres may represent axons from adjacent ganglia. Furthermore, two types of nerve fibres have been seen to innervate the muscle layer ChAT/NF⁺ and ChAT/NF⁻ fibres. The ChAT/NF⁺ fibres may also contact interstitial cells in the outer muscle layer. Thus, there is evidence for a sub-urothelial ganglionic network with the potential to receive inputs from the urothelium and adjacent ganglia, which has an output to the muscle layers. It has been speculated that such a network is involved in the local bladder wall reflexes linking urothelial distortion to phasic contractile activity (Gillespie et al, 2006a). The sub-urothelial ganglion cells receive inputs from two types of nerve fibre that are likely to be cholinergic suggesting that the ganglionic cells express cholinergic receptors. Therefore, the mediation of ganglionic activity is likely to have a strong cholinergic influence, with application of exogenous muscarinic agonists activating these ganglia. Thus, there is circumstantial evidence to support a role for the suburothelial ganglionic network, the pacemaker mechanism, and the different types of interstitial cells; resulting in a heterogeneous network carrying pacemaker activity to the muscle layer.

The present observations, derived from the isolated guinea pig bladder, show that the rapid component of the phasic activity is generated and its frequency determined by activation of muscarinic M₃ receptors. This pacemaker activity is particularly sensitive to the M₃ specific antagonist 4-DAMP at sub-nM concentrations; concentrations

unlikely to affect the micturition contraction. Therefore, it is suggested that a mechanism is present that may act as a target for low doses of M₃ selective antagonists. Non-micturition activity has been described both in normal human subjects and in patients with OAB (Gillespie, 2005b; Mosso and Pellacani, 1882; Bristow and Neal, 1996; Heslington and Hilton, 1996; Van Os-Bossagh et al, 2001; Drake et al, 2005); and, as previously discussed this activity may be involved in the modulation of bladder sensation (Gillespie, 2005b). On this basis, the tentative suggestion is made that the antimuscarinic drugs exert their therapeutic action, either wholly or in part, through the inhibition of pacemaker activity within the bladder wall which in turn is involved in the generation of non-micturition bladder sensation.

4.3 Intrinsic characteristics of the volume response

As shown previously, changes in intravesical volume lead to changes both in frequency and amplitude of spontaneous phasic activity (Drake et al, 2003a and 2003c), with each transient contraction associated with a burst of afferent discharge (Andersson and Yoshida, 2003; Vaughan and Satchell, 1995). Since phasic activity is considered to be part of an integrated motor/ sensory system, the responses in activity to volume suggest that the system receives inputs directly from, or related to, intravesical volume. In addition, it has also been shown that phasic activity can receive further excitatory and inhibitory inputs (Gillespie, 2004a; Gillespie and Drake, 2004b), and as such afferent discharge related to volume could be influenced by these other stimuli. Therefore, sensory perception of intravesical volume has the capacity to be modulated by mechanisms acting in the periphery (Lagou et al, 2004).

It has been previously proposed (Lagou et al, 2004) that a number of complex arrangements exist within the bladder that could account for the physiological responses seen within phasic and volume related activity. The excitatory effect of substance P and the inhibitory effect of calcitonin gene related peptide (CGRP), (Gillespie, 2005a), upon phasic activity suggest the presence of a sensory-axon collateral reflex within the bladder wall; supporting a role for the release of sensory neurotransmitters within the wall of the bladder. Certainly, it has been documented previously that afferent nerves can send collateral fibres to structures within the tissue where they originate (Maggi, 1990; Maggi and Meli, 1986 and 1988). However, with prolonged exposure to capsaicin, leading to the elimination of sub P/ CGRP sensory nerves (Maggi and Meli, 1998), volume induced changes are not affected. This would suggest that the local sensory-collateral reflex is not involved in the volume response.

Additionally, since nearly all potential neural sensory influences would be removed via the action of capsaicin, non-neural mechanisms must be considered to be involved in the regulation of volume induced activity.

The urothelium is known to release adenosine triphosphate (ATP) in response to mechanical distortion (Ferguson et al, 1997). It has been suggested that the role of urothelial released ATP is to modulate firing of afferent nerve fibres, in turn influencing bladder sensation (Ferguson et al, 1997; Birder et al, 1998; Lee et al, 2000). However, it has been further suggested that ATP can indirectly act upon the bladder via alterations in phasic activity (Gillespie, 2004), inducing sensory neurotransmitter release via anti-dromic activation of collateral afferent nerve fibres (Lagou et al, 2004). The effect upon volume induced inhibition, however, has not as yet been assessed.

Further potential sources of non-neuronal mechanisms include interstitial cells and a non-specific substance termed 'inhibitory factor'. It has been postulated that interstitial cells have a role in modulation of volume induced activity by inhibiting phasic activity following a volume reduction; since ODQ, an inhibitor of guanylate cyclase, has been found to suppress this inhibition (Lagou et al, 2004). Whilst 'inhibitory factor' is speculated to be released from the urothelium resulting in an inhibition of detrusor contraction (Hawthorn et al, 2000). However, the results upon phasic activity have not been assessed.

4.4 Effects of alterations in intravesical volume, duration of volume increase and agonist concentration

The observations described in these groups of experiments illustrate the responses of the isolated guinea pig bladder to changes in intravesical volume, duration of volume increase and the effects of the two doses of arecaidine, 100 and 300nM, upon volume induced 'augmented' activity ('volume response'). As previously shown a 'steady state' is seen in phasic activity following prolonged exposure to arecaidine. The effects of the volume response were assessed after a period of equilibration after addition of agonist to allow for the experiments to be consistently performed during this 'steady state'. Following an increase in intravesical volume a complex series of events are seen similar to those described following the addition of arecaidine; again an 'initial phase' consisting of a burst high frequency, low amplitude transients followed by a 'steady state' consisting of regular transient contractions of a lower frequency but greater amplitude. Following a reduction in intravesical volume a period of quiescence is seen with transient contractions gradually returning to baseline frequency and amplitude.

The 'initial phase' seen following volume increase comprises a succession of rapid, small amplitude transient contractions superimposed upon an initial sudden rise in underlying basal pressure followed by a gradual decline. The overall magnitude of this 'initial' response is dependant on the increase in intravesical volume, illustrated by the instantaneous frequency (IF_{init}) and underlying pressure (P_{shift}) being significantly raised in comparison between a 2.0ml than 1.5ml increase. However, as P_{shift} falls to 'steady state' levels, frequency also gradually reduces with the amplitude of transients increasing. As discussed, the magnitude of the 'initial phase' seems to be directly

related to the magnitude of the increase in intravesical volume, as shown by the differences between 2.0 and 1.5ml increases. Additionally, when raw data is analysed from previously published records illustrating the effects of smaller sequential increases in volume the initial phase, the initial phase is found to be smaller (Lagou et al, 2004). However, the final 'steady state' between our data and that of Lagou, at comparable volumes, remains similar suggesting that the 'steady state' is unaffected by rate of volume increase. Furthermore, under physiological conditions an 'initial phase' is not seen, though a gradual increase in frequency, amplitude and underlying pressure is apparent (Streng et al, 2006), similar to that seen in the steady states at increasing volumes during our experiments. Therefore, the burst of activity seen immediately following a rapid increase in volume is likely to represent a non-physiological response in the normal bladder. However, it does illustrate that the mechanisms involved in the regulation of pacemaker frequency have difficulty compensating immediately for the effects of increased filling rates, essentially becoming 'overloaded'. The reasons for this are unclear. However, one possible explanation is that a rapid efflux of acetylcholine (ACh) from the urothelium in response to stretch occurs that preferentially activates a sensitive M_3 component of pacemaker activity. Giving an agonist induced initial 'burst' of activity in a manner analogous to that described in the previous section. Though this phenomenon is non-physiological, further investigation may give insight into the mechanisms involved in the generation and regulation of volume induced spontaneous activity.

Following volume reduction a period of quiescence is seen termed the 'inhibitory phase'. This has been noted both *in vitro* (Lagou et al, 2004) and *in vivo* (Streng et al, 2006); suggesting this to be a true physiological response as opposed to a factor related

to increased fill rates (Streng et al, 2006). The experiments described here further illustrate the 'inhibitory phase' to be influenced by a number of factors consisting of volume increase, duration of increase, concentration of muscarinic agonist and degree of bladder emptying. The length of the inhibitory phase increases with volume, however, this is not entirely dependant upon the absolute change in volume increase. It has been shown that the length of the inhibitory phase is also dependant upon volume decrease, with longer inhibitory phases being seen when volume returns to baseline. This is highlighted when the mean results of a volume increase and decrease of 1.5ml (157 seconds) are compared with those following a volume increase of 2.0ml and decrease of 1.5ml (118 seconds). From this simple observation it may be argued that a two stage process is evident in the regulation of the inhibitory phase. Volume increase may act as a volume dependant stimulus for the synthesis of agents, or initiation of mechanisms, involved in the inhibitory phase that are only fully released/ triggered again in a volume dependant manner as the bladder empties. The relationship between the magnitude of bladder emptying and the length of the inhibitory phase would also suggest that the stimulus of volume decrease is of similar importance to that of volume increase in the determination of the length of the inhibitory phase. It can be further argued that an increase in the inhibitory phase following a return to baseline volume would suggest that maximal mechanical distortion, as opposed to pure stretch, is required for the full effects of the inhibitory response to be seen. Whether this is predominantly related to specific structures is open to speculation, however, it may indicate a role for the urothelium since this may be indicative of the urothelium unfolding as the bladder fills and re-folding as it empties. ATP and NO released from the urothelium upon unfolding/ re-folding could easily exert an effect upon suburothelial purinergic receptors (Ferguson et al, 1997; Birder et al, 1998; Lee et al,

2000) or interstitial cells (Lagou et al, 2004). Certainly, suburothelial interstitial cells seem to be implicated in some way to the inhibitory phase since ODQ, an inhibitor of guanyl cyclase and therefore cells signalling via cGMP, significantly reduces its length. Since in the mouse/ guinea pig interstitial cells are the predominant cells acting via the NO/ cGMP pathway this would implicate interstitial cells as being directly involved in either its generation or co-ordination.

Duration of volume increase has also been shown to play a role in the length of the inhibitory phase, with longer durations of fill being associated with longer inhibitory phases. An insignificant difference in the length of the inhibitory phase is seen following durations of fill less than 30 seconds, with a small but significant difference seen between the durations of fill of 100 and 500 seconds. However, a substantial difference is seen following a 30 and 100 second volume increase; suggesting that between these two time points the rate of increase in the initiation of these mechanisms involved in the regulation of the inhibitory phase rise substantially. The relationship between the inhibitory phase and duration of fill further supports the idea of agents/ mechanisms involved in the inhibitory phase being synthesised/ initiated in response to volume increase. It also supports the inhibitory phase to be a true physiological response as opposed to flaccidity related to stretch induced damage of the underlying detrusor muscle. Further supporting the idea that quiescence following volume reduction is related to inhibition of either pacemaker function or co-ordination of activity and not due to a direct relaxant effect on the detrusor is that during the inhibitory phase the bladder is still able to contract in response to electrical field stimulation, (Lagou et al, 2004).

The response of phasic activity in the isolated bladder to alterations in intravesical volume is complex. However, the effects illustrated following addition of 100 and 300nM arecaidine suggest a strong cholinergic input, involved in the modulation of all the three stages (initial, steady state and inhibitory phase); with cholinergic inputs exerting an excitatory effect by acting directly on the pacemaker mechanism. This increased pacemaker stimulus results in an increase in the instantaneous frequency following volume increase (IF_{init}) and frequency at steady state (F_{ss}). However, it also leads to a reduction in the inhibitory phase. This suggests the mechanisms involved in the generation of the inhibitory phase to involve a balance involving both inhibitory and excitatory stimuli, with an excitatory cholinergic component. Since the factors involved in the inhibitory phase are unlikely to directly involve the detrusor, the likely targets are again likely to be those mechanisms involved either the generation or co-ordination of spontaneous activity; either the pacemaker ($M_3 > M_2$) or co-ordination ($M_2 > M_3$).

From these observations it has been speculated that the regulation of volume induced spontaneous activity and modulation of the inhibitory phase relies upon a complex series of excitatory and inhibitory inputs. Rapid increase in intravesical volume leads to a burst of increased frequency that gradually declines, suggesting the presence of a relatively rapidly adapting mechanism involved in the modulation of volume related activity that becomes more pronounced at non-physiological rates of bladder filling. Rapid reduction in intravesical volume leads to a quiescent period, termed the inhibitory phase. This is related to both the duration of intravesical volume increase and its magnitude. However, similar volume changes are more effective when volume is reduced back to baseline as opposed to the bladder being incompletely emptied.

Furthermore frequency of transient contractions remains constant once steady state has been reached with no evidence of inhibition pre-volume reduction. These functional observations suggest that mechanisms involved in the generation of the inhibitory phase initiate during bladder filling, requiring a period of time >30seconds to exert a significant effect. However, they depend upon a reduction in volume to be triggered, with the response dependant upon the volume reduced. The mechanisms involved in the generation and modulation of the inhibitory phase seem to be regulated by a strong cholinergic input, however, the exact nature of these mechanisms are unknown. Certainly structures have been identified, in the form of interstitial cells and ganglia, capable of generating and modulating such activity. Furthermore, their close proximity to the urothelium which is capable of releasing ATP, NO and ACh in response to stretch strongly suggests a potential role for the urothelium in the transduction of such activity.

4.5 Effects of Tolterodine upon volume induced changes.

Tolterodine is an orally administered anti-muscarinic drug commonly used in the treatment of OAB. It is a competitive muscarinic receptor antagonist with a relative selectivity for M₂ receptors over M₃ (Nelson et al, 2004), though its efficacious effects are regarded to be derived from its increased affinity for muscarinic receptors found in the bladder over those in the parotid gland (Nelson et al, 2004). Clinical trials have shown it to be effective in reducing symptoms of frequency, urgency and urge related leakage (Chapple et al, 2005). Although, at clinical doses there is no significant reduction in the strength of detrusor contraction (Finney et al, 2006), higher doses result in a significant reduction associated with a marked increase in residual volume (Stahl et al, 1995).

The observations reported in this series of experiments illustrate the response of increasing doses of Tolterodine upon volume induced spontaneous activity in the isolated bladder, augmented by 100nM arecaidine. Compared to controls no significant effect was seen following addition of 10nM Tolterodine. Doses of 30nM or more were required before any significant effect was seen. These included a dose dependant reduction in frequency immediately following volume increase (IF_{\max}) and the frequency at steady state (F_{ss}), in addition to an increase in the inhibitory phase. As discussed previously the mechanisms responsible for generating pacemaker activity during IF_{\max} and regulating frequency during the steady state, F_{ss} , are likely to have a cholinergic input; as increasing doses of muscarinic agonist increase both these parameters. The observations seen with Tolterodine further support this suggestion. The inhibitory phase (IP) also seems to be regulated, in part, by a cholinergic input. As suggested previously, the inhibitory phase is modulated by both competing excitatory and inhibitory inputs acting upon pacemaker activity. This data would suggest that the mechanisms responsible for the excitatory component are cholinergic.

This data further supports the importance of cholinergic mechanism in the regulation of volume induced spontaneous activity. Although the locations of these mechanisms remain unknown they seem to exert a direct excitatory effect upon pacemaker function, giving rise to an increase in IF_{\max} and the frequency at steady state; in addition to promoting the return of activity during the inhibitory phase. With the relative lack of selectivity of Tolterodine between the M_2 and M_3 muscarinic subtypes no weight can be given to the relative roles of either of these receptors in the modulation of volume induced activity. However, it does further illustrate potential mechanisms that may be involved to account for the clinical effects of anti-cholinergics.

4.6 Effects of the nicotinic antagonists hexamethonium and pancuronium on volume induced activity

It has been suggested previously that the regulation of both autonomous activity and volume induced activity consists of a number of competing excitatory and inhibitory inputs. Though the location of these mechanisms and inputs are unknown, structures have been identified with the potential of generating and co-ordinating these competing signals. In particular, ganglia have been identified close to, and receiving nerve fibres from, the urothelium that synapse with the interstitial cells found around the detrusor. Some of these ganglia have been found to consist of nerve bodies containing choline acetyl transferase (ChAT) and calcitonin gene related peptide (CGRP). As shown during these experiments cholinergic input exerts an excitatory effect upon spontaneous activity, whilst CGRP has been found to be inhibitory (Gillespie, 2005a). As the functional roles of ganglia in relation to the regulation of spontaneous and volume induced activity are previously unknown the effects of the nicotinic receptor antagonists, hexamethonium and pancuronium bromide, were assessed.

These experiments illustrate the effects of hexamethonium, a nicotinic antagonist selective for receptor subtypes found on autonomic ganglia, and pancuronium, a neuromuscular nicotinic receptor antagonist (Fryer and MacLagan, 1987; Maestrone et al, 1994; Lowenick et al, 2001), upon spontaneous and volume induced activity augmented by 100nM arecaidine. Hexamethonium had no effect upon either the frequency of transient contractions prior to volume increase, immediately following increase (IF_{max}) or at steady state (F_{ss}). On the assumption that these ganglia express ganglionic nicotinic receptors, the lack of an effect seen following addition of

hexamethonium suggests that regulation of the frequency of transient contractions during filling is unrelated to mechanisms directly involving ganglia. It could also be argued that if nicotinic receptors were present upon the pacemaker mechanism, they would not be of 'ganglionic' origin.

The effect of hexamethonium in prolonging the inhibitory phase suggests a role for ganglia in co-ordinating the return of phasic activity. It has been previously argued that the 'inhibitory phase' derives from a transient inhibition of either the pacemaker or the mechanisms co-ordinating spontaneous activity, and that these can be modulated by both excitatory and inhibitory elements. As an antagonist, hexamethonium acts as an antagonist it must act through inhibition of an excitatory, nicotinic mediated receptor. This is unlikely to exist directly upon the pacemaker mechanism itself otherwise a decrease in frequency would be seen upon direct application of hexamethonium. The excitatory reflex mechanism whereby volume regulates frequency is also unlikely to be directly effected since no change was seen in either the IF_{max} or F_{ss} . Therefore, hexamethonium must be acting on a mechanism distinct from the 'volume/ frequency' reflex, at a point not directly related to the pacemaker mechanism. This would support the idea of a ganglionic network involved in co-ordinating the return of activity during the 'inhibitory phase', especially since there is structural evidence to support the presence of ganglia.

An alternative mechanism to be considered is the role of the urothelium in this activity. mRNA for the nicotinic subunits $\alpha 3$, $\alpha 5$, $\alpha 7$, $\beta 3$, $\beta 4$ have been detected in rat urothelium by RT-PCR, with $\alpha 3$ and $\alpha 7$ also being confirmed upon Western blotting

(Beckel et al, 2006). In cultured cells, addition of nicotine results in an increase in intracellular calcium concentration suggesting that these subunits form functionally relevant receptor channels. This activity has also been shown to be blocked by hexamethonium, suggesting the presence of a functional hexamethonium sensitive nicotinic receptor expressed upon the urothelium. Thus, it is conceivable that the increase in the inhibitory phase seen with hexamethonium may occur, to some extent, through inhibition of the release of excitatory mediators from the urothelium during bladder emptying.

In contrast to the effects of hexamethonium, pancuronium was found to exert an effect on both frequency and the inhibitory phase. The effects on frequency following application of pancuronium in addition to IF_{\max} and F_{ss} , may suggest a direct input of a pancuronium sensitive nicotinic receptor to the pacemaker mechanism. Furthermore, a similar nicotinic receptor may be present in the mechanisms co-ordinating the return of activity during the inhibitory phase. However, a degree of caution must be exercised in the interpretation of these results as it is acknowledged that pancuronium has an anti-muscarinic effect at higher doses, effecting both M_2 and M_3 subtypes (Hou et al, 1998). Though these effects may represent, in part, non-specific muscarinic responses it does potentially highlight the presence of nicotinic mechanisms within the bladder. Especially when the effects of Lobeline, a nicotinic ligand, are also considered (discussed previously). Whether this represents different nicotinic receptor subtypes, or one with a binding affinity for both hexamethonium and pancuronium further work is required to pinpoint their location and subunit composition.

These observations illustrate a functional role for nicotinic receptors in the regulation of volume induced spontaneous activity. Those sensitive to hexamethonium are likely to be excitatory, co-ordinating the return of activity during the inhibitory phase. They do not seem to have a direct input to the pacemaker mechanism and seem to be distinct from the mechanism whereby volume regulates frequency; supporting a role for both ganglia and a nicotinic mediated urothelial response. The effects of pancuronium are less certain and may represent non specific cholinergic effects. However, pancuronium sensitive nicotinic receptors may be present both on the pacemaker mechanisms and the excitatory mechanisms during the inhibitory phase. Whether these hexamethonium and pancuronium sensitive receptors are distinct nicotinic subtypes, or the same receptor sharing some binding affinity for both drugs is open to speculation. However, it does highlight the need for further work in order to identify and evaluate the nicotinic mechanisms within the bladder.

4.7 Effects of α/β methylene ATP on volume induced activity

The data obtained during the course of these experiments illustrate a number of observations that highlight potential novel roles for adenosine triphosphate ATP. The addition of α/β methylene ATP (α/β MATP), a non-hydrolysable form of ATP, to the isolated bladder resulted in a complex series of excitatory and inhibitory effects upon phasic activity; suggesting ATP to be a potent modulator of the mechanisms regulating pacemaker function.

ATP has been found to exert an effect directly upon smooth muscle, leading to non-cholinergic detrusor contraction. It is currently believed that ATP released from nerves, by itself or in combination with acetylcholine (ACh), acts to depolarise the muscle via

P2X purinergic receptors located on the detrusor smooth muscle (Inoue and Brading, 1990; Fry et al, 2002). In addition there is also evidence to suggest a sensory role for ATP in the generation of afferent activity from the bladder (Cook and M^cCleskey, 2000), probably acting via P2X₃ receptors located upon suburothelial sensory C-fibres (Lee et al, 2000). The combination of one, or both, of these ATP-dependant components have been implicated in the pathological conditions, bladder hypertrophy (Calvert et al, 2001), idiopathic detrusor overactivity (Bayliss et al, 1999), and interstitial cystitis (Palea et al, 1993).

The effect of α/β MATP upon spontaneous phasic activity in the isolated guinea pig bladder has been recently described under iso-volumetric conditions (Gillepie, 2004d). The effects upon both augmented and un-augmented bladders in increasing phasic activity, in addition to underlying contraction, suggest ATP to exert its influence on the regulatory mechanisms controlling phasic activity, and not just upon the detrusor. Addition of α/β MATP to the bladder preparation results in a rapid burst of frequency superimposed upon a rise in underlying pressure that gradually declines as underlying pressure falls. This effect has been previously documented by Gillespie, but has been confirmed during the course of these experiments. Following washout of α/β MATP from the preparation a profound inhibition in activity is seen that gradually returns; similar in nature to the 'inhibitory phase' seen following a volume response. Together, these two observations suggest the presence of two functionally relevant purinergic receptors with direct inputs to the mechanism regulating pacemaker activity. One excitatory, with a rapid onset, and the other inhibitory, with a slower onset. However, when both are stimulated the overall, net effect is excitatory, suggesting the excitatory mechanism to be dominant.

The observations made during the course of these experiments further illustrate the effects of α/β methylene ATP upon the volume induced activity of the isolated guinea pig bladder augmented by 100nM arecaidine. Volume responses performed in the presence of both 100 and 300nM α/β MATP show an exaggerated increase in frequency both immediately after volume increase (IF_{max}) and at steady state (F_{ss}), compared to baseline frequency pre-volume increase. This discrepancy between IF_{max}/F_{ss} and baseline frequency seems to be far more pronounced in the presence of α/β MATP compared to the other compounds studied. So much so, that it may be putatively speculated that ATP plays a role in the mechanism whereby volume regulates frequency. In addition to the excitatory effect upon IF_{max} and F_{ss} , α/β MATP also exerts an inhibitory effect upon volume induced activity by prolonging the inhibitory phase. With α/β MATP exerting a direct excitatory effect upon pacemaker activity, the quiescence seen during the inhibitory phase suggests a degree of purinergic mediation in the mechanisms that suppress pacemaker activity during this time.

The exact locations of the mechanisms through which α/β MATP may be acting, or the purinergic receptor subtypes involved, are unknown. It is generally accepted however, that P2X receptors exert excitatory actions on bladder smooth muscle (McMurray et al, 1998), and it is speculated that these may also be involved in the excitation of pacemaker activity. Conversely, inhibition of pacemaker activity may be mediated by P2Y receptors. P2Y receptors have been shown to be coupled to adenylyl cyclase, stimulation of which leads to an increase in cAMP. Experiments with forskolin, an activator of adenylyl cyclase, have shown a slowing of phasic activity (Gillespie, 2004d), indirectly supporting a potential inhibitory effect of P2Y receptors.

The results from these experiments, using α/β methylene ATP alone, have illustrated a number of novel roles for ATP in the regulation and modulation of spontaneous and volume induced activity. ATP seems to display a dual response by exerting an excitatory effect upon pacemaker activity in addition to an inhibitory effect through the potentiation of the inhibitory phase. This would suggest the presence of two or more functionally relevant purinergic receptors acting at various locations in the control and regulation of spontaneous activity. Though these locations are currently open to speculation, suburothelial afferent nerves lying in close proximity to the suburothelial interstitial cell network have been found to display P2X3 receptors (Lee et al, 2000), stimulation of which are thought to be related to the generation of sensation.

4.8 Effects of the purinergic receptor antagonists NF 279, MRS 2179 and PPADS upon volume induced activity

These experiments illustrate the effects of the purinergic receptor antagonists NF 279, MRS 21709 and PPADS upon spontaneous volume induced activity augmented by 100nM arecaidine. The individual effects of these antagonists were complex and variable, showing that the frequency immediately following application of agonist and prior to volume increase, in addition to the frequency immediately after volume increase (IFmax), frequency at steady state (Fss) and the inhibitory phase to be altered in different ways by specific antagonists. These observations illustrate that a number of functionally relevant purinergic receptor subtypes to be present upon the mechanisms responsible for the generation and modulation of spontaneous and volume induced activity; inducing direct effects upon the pacemaker and the inhibitory phase.

PPADS is a non specific P2 purinergic receptor antagonist addition of which resulted in a significant increase in the frequency of transient contractions throughout the volume response in addition to markedly reducing the inhibitory phase. In itself these observations suggest the net effect of P2 receptor stimulation to be inhibitory, leading to a direct inhibition of the pacemaker mechanism and excitation of the mechanisms involved in the inhibitory phase. However, in conjunction with the effects of the α/β methylene ATP data there seems to be a discrepancy between the effects of purinergic receptors upon pacemaker activity. It is difficult to account for the increase in the frequency of transient contractions seen by both a purinergic agonist and antagonist, and therefore this may represent a pharmacological 'anomaly' of one of the compounds.

Given the location and properties of the suburothelial interstitial cells it is postulated that they may represent part of the regulatory mechanisms involved in either the generation or modulation of pacemaker activity. They are known to express purinoceptors, predominantly P2Y₆ (Sui et al, 2006), and generate spontaneous intracellular Ca²⁺ transients in isolated suburothelial interstitial cells in response to ATP (Wu et al, 2004). However, Wu et al also described that α/β methylene ATP had no effect upon these cells suggesting that their expressed purinoceptors are not ionotropic and may be part of the P2Y family, confirmed later by Sui et al. The lack of an effect of α/β methylene ATP on non-ionotropic P2Y receptors would certainly account for the observations seen by ourselves; with α/β MATP exerting a preferentially excitatory effect upon P2X receptors without the inhibitory effect of P2Y, and PPADS exerting an effect upon both receptor subclasses. These observations would therefore suggest that

the suburothelial interstitial cells are capable of exerting an inhibitory influence in the modulation of pacemaker activity. Their expression of P2Y receptors may also indicate this mechanism to involve induction of cAMP by stimulation of adenylyl cyclase.

The actions of both α/β methylene ATP and PPADS upon the inhibitory phase suggest a direct purinergic input into the mechanisms responsible for its generation. However, given the potential relative sites of action of these two compounds, highlighted above, the inhibitory phase must comprise of a number of purinergic mediated responses that involve a balance of both excitatory and inhibitory stimulation.

NF 279, a relatively specific antagonist for P2X₁ receptors, and MRS 2179, a relatively specific P2Y₁ antagonist, were both found to significantly reduce the frequency of transient contractions immediately after application to the organ preparation in addition to significantly increasing the frequency immediately after volume increase (IF_{max}). This would suggest that these receptors exert a direct excitatory effect upon the mechanisms involved in the modulation of frequency, or on the pacemaker mechanism, in addition to inhibiting the rapidly adapting mechanism that increases frequency along with volume (illustrated by IF_{max}). Alternatively, as illustrated earlier, the mechanisms involving purinergic responses seem to be complex. It is therefore conceivable that their actions are indirect, by altering the balance of excitatory/ inhibitory stimuli.

At steady state following a volume increase, NF and MRS were found not to display any significant effect upon the frequency of transient contractions (F_{ss}). However, they were found to significantly affect pacemaker activity at 'baseline' volume. This would

suggest that P2X₁ and P2Y₁ receptors are actively involved in the modulation of pacemaker activity at low volumes, but exert less, or any, influence at higher volumes. The effects of PPADS upon frequency at steady state (F_{ss}) would suggest the presence of functionally relevant purinoceptors at higher volumes, arguing that purinoceptors are involved in the regulation of frequency throughout bladder filling. Although the effects of PPADS are non-specific it would suggest that purinoceptors other than P2X₁ and P2Y₁ become more functionally relevant as the bladder fills. Therefore, it is conceivable that the functional relevance of purinergic receptor subtypes are related to volume; although it cannot be speculated whether this would be related to receptor desensitisation or their related corresponding mechanisms. Additionally, the effects upon the inhibitory phase would suggest an involvement of P2X₁ receptors, but not P2Y₁.

The effects of NF 279, MRS 2179 and PPADS suggest a complex interaction of functionally relevant purinoceptors subtypes throughout bladder filling, affecting the mechanisms involved in the generation and modulation of spontaneous and volume induced activity. The location and nature of these receptor subtypes are unknown; however, there is indirect evidence to speculate that suburothelial interstitial cells may be involved in this modulation in exerting an inhibitory affect. It may also be speculated that the functional relevance of a purinoceptor subtype is volume dependant; though whether this is a reflection of receptor desensitisation or the underlying mechanism involved is not known.

4.9 Summary

As with all animal experiments caution needs to be exercised when extrapolating data to the human. However, given the relative inaccessibility of isolated human bladders there is a necessity to perform functional experiments upon the more accessible animal models. There are always going to be differences between the animal species, some subtle others large, but if attempts are made to relate the structure of each animal model to function this would further our understanding of the physiological mechanisms within the human bladder. Although the experiments performed as part of this thesis were upon the isolated guinea pig bladder a degree of anatomical and physiological similarity between the guinea pig and human bladder is evident. Both consist of distinct layers consisting of urothelium, lamina propria and underlying detrusor. More importantly many of the structures that have been postulated, as part of this thesis, as playing a part in the generation and modulation of spontaneous activity in the guinea pig are also present in human bladder. Smet et al, 1996 demonstrated the presence of cells analogous to the Interstitial Cells of Cajal present within the sub-urothelial and detrusor layers of both the guinea pig and human, with both sets of cells from each species responding to stimulation with nitric oxide with a rise in cGMP. They have also demonstrated the presence of ganglia within the sub-urothelial and detrusor layers. Other work has demonstrated a similar ratio of M_3 / M_2 receptors within the two different bladders with M_3 receptors being involved in the mediation of contraction for both, (Wang et al, 1995). Furthermore, the response of the detrusor to purinoceptor activation leads to similar responses, (Inoue and Brading, 1991).

However, the data evident for the similarities between the human and guinea pig relates to individual specific responses and phenomenon. Little to no data is available

regarding the integrative physiology for both species. Although similarity exists regarding the anatomy and structure of the two bladders, insofar that certain cell types are present in both species, the data presented as part this thesis illustrates the 'sensitivity' of the mechanisms involved in the regulation of spontaneous activity. As such, a small variation in a single response or a different interaction between responses could affect the overall integrative function. However, given the basic anatomical similarities between the two species the isolated responses are likely to have some, if only a minor, degree of similarity. Unfortunately, the similarity between the guinea pig and the human can only be fully justified once the structures of both have been more fully assessed, in addition to cataloguing the structure and function of a number of other species.

Spontaneous and volume induced phasic activity in the isolated guinea pig bladder is generated and modulated by a complex interaction of opposing excitatory and inhibitory stimuli. Although the exact nature and location of these mechanisms are not known, they represent local mechanisms inherent to the wall of the bladder. Activity similar to the phasic transient contractions seen in the guinea pig have been described in human subjects both in the 'normal' bladder and in those suffering from overactive bladder syndrome (OAB) (Gillespie, 2005b; Mosso and Pellacani, 1882; Bristow and Neal, 1996; Heslington and Hilton, 1996; Van Os-Bossagh et al, 2001; Drake et al, 2005). Furthermore, it has been suggested that phasic activity represents a component of afferent activity involved in the generation of sensation; potential dysfunction of which could be a factor in the development of OAB (Gillespie, 2005b). This system involving direct motor/ sensory interaction leading to potential cholinergic modulation of sensory afferents has therefore given rise to a mechanism upon which antimuscarinic

medication may act. The importance of this finding is especially relevant in light of retrospective analysis of older data suggesting antimuscarinic medication currently used in clinical practice has little, if any, significant effect upon contraction, instead acting on the afferent side by reducing sensations of filling (Finney et al, 2006); an affect that could not be accounted for by the classical description of bladder physiology. Further evaluation of the effects of the M_3 specific muscarinic antagonist 4-DAMP have identified the initial response of the bladder to muscarinic stimulation to be particularly sensitive to the effects M_3 antagonism. Especially at sub-nM concentrations, insufficient to generate detrusor contraction. It is therefore suggested that the effects of the antimuscarinic drugs exert their therapeutic action, either wholly or in part, through the inhibition of mechanisms related to pacemaker activity within the bladder wall which are involved in the generation of non-micturition bladder activity.

The potential importance of spontaneous activity as a therapeutic target is not restricted to antimuscarinic medication. The intrinsic characteristics of spontaneous, phasic activity in response to volume have highlighted induced changes that comprise of an inhibitory phase whereby pacemaker activity, and therefore the frequency of transient contractions, becomes quiescent for a period of time before gradually returning to normal. This 'inhibitory phase' is dependant upon the duration of bladder filling and the volume by which it has been increased and decreased. A volume change above and back to baseline is far more effective in prolonging the inhibitory phase than a similar volume change not returning to baseline volume. Suggesting that the inhibitory phase is dependant upon the time and magnitude of the volume increase for the initiation of the mechanisms potentially to be involved, in addition to being reliant upon maximal

volume decrease (or alternatively maximal mechanical distortion of the bladder) for these mechanisms to be fully triggered. The physiological relevance of this is not entirely known, however, it is postulated that a decrease in afferent activity during micturition could reduce sensation that otherwise may be uncomfortable or even painful. From the clinical perspective further understanding of the mechanisms behind the 'inhibitory phase' may give rise to further therapeutic targets; manipulation of which may be able to induce artificial periods of quiescence.

The duration of the inhibitory phase following volume decrease has been found to be altered by antimuscarinic agents, nicotinic antagonists and purinergic agonist/antagonists suggesting its underlying generation and regulation to be complex. Though, this data does suggest direct cholinergic and purinergic inputs into the modulation of the inhibitory phase with ganglia playing a role in the co-ordination of the return of activity during this time. In relation to current practise the 'inhibitory phase', occurring following bladder emptying and being related to the volume reduction, raises an interesting point regarding standard, non-ambulatory urodynamic investigations. Although its significance *in vivo* is not known it does suggest that a reduction in sensation may occur following voiding. As voiding prior to artificial filling is standard practise during urodynamic assessment, it is conceivable that filling during the 'inhibitory phase' may involve filling during a period of sensory quiescence giving rise to aberrant results.

In addition to the role of artificial filling following micturition, the rates of filling during conventional urodynamics also need to be questioned. These experiments show,

albeit at the extremes of fill rate, that the mechanisms relating volume to spontaneous activity are unable to adapt immediately to non-physiological rates essentially becoming 'overloaded', though given a short period of time can eventually adapt. The ability of the bladder to adapt to higher rates of fill has also been questioned in humans. When the results of ambulatory (physiological fill rates) and conventional urodynamics (non-physiological fill rates) have been compared a reduction in spontaneous activity can be seen with conventional studies, in addition to a comparative rise in the volume threshold for micturition and an impairment in detrusor contractility, Robertson et al, 1994. Furthermore, underlying rise in intravesical pressure is more marked with higher fill rates (lower compliance) and negligible at physiological fill rates (almost completely compliant), giving rise to the argument that bladder compliance is strongly related to, if not a factor of, the rate of bladder filling, Klevmark 2002. Though, it has been previously argued that the differences seen between conventional and ambulatory urodynamics need to be explained at the cellular level, Klevmark 2002, the exact nature these have not been described. However, these experiments give some insight into the mechanisms that could account for these findings.

The effects of hexamethonium and pancuronium have together suggested the presence of nicotinic receptors involved in the modulation of spontaneous and volume induced activity. The effects of hexamethonium suggest the presence of functionally relevant ganglia, whilst the effects of pancuronium suggests the presence of nicotinic receptors more akin to those found upon skeletal muscle. This was not a predicted finding as pancuronium was not thought to exert an effect upon smooth muscle. Therefore, there is the potential for the presence of a nicotinic receptor with similar binding characteristics to those found upon skeletal muscle, which may act as a useful target if

unique, or predominantly located to, the bladder. Certainly, the presence and nature of nicotinic receptors within the bladder needs to be addressed further, both in structure and function.

Purinoceptors were found to display a varied and complex response, both on pacemaker activity and upon the inhibitory phase. Both excitatory and inhibitory receptors were present; however, individual subtypes (P2X₁ and P2Y₁) seemed to display a differing degree of functional relevance at low and high volume. Though P2X and P2Y may have excitatory and inhibitory effects respectively, the overall effect of purinergic receptor stimulation is inhibitory, as suggested by the actions of PPADS. The locations of the mechanism involved in the modulation of spontaneous activity and the generation of the inhibitory phase are not known. However, there is the suggestion that the overall purinergic effect on the pacemaker effect is inhibitory, involving a balance of excitatory and inhibitory purinergic stimuli. Some have postulated that myofibroblasts are implicated in an inhibitory P2Y₆ response, Sui et al, 2004, however, though these cells are spontaneously active their relationship to the suburothelial interstitial cell layer is not clear. However, the marked effects of the purinergic antagonists upon spontaneous activity would suggest that purinoceptors may act as useful therapeutic targets.

FURTHER STUDY**Chapter Five**

- (i) The effects of 4-DAMP and AF-DX need to be repeated upon the volume response, to elicit the relative responses of the M_2 and M_3 muscarinic receptor subtypes at various stages of filling, and upon the 'inhibitory phase'. It could then be seen whether the M_3 mediated rapid, initial component of cholinergic activity (under iso-volumetric conditions) also mediates the initial, rapid component seen immediately following volume increase.
- (ii) The nicotinic responses within the bladder need to be considered further. Effects of nicotinic agonists need to be assessed upon spontaneous and volume induced changes, in addition to more specific nicotinic antagonists. Also, the location of nicotinic receptors needs to be evaluated in addition to the subtypes involved. This may involve a combination of immunohistochemical staining techniques, in addition to the mRNA analysis of specific structures.
- (iii) As the purinergic responses seem to be complex, potentially involving a number of different subtypes from both the P2X and P2Y family, the effects of the specific purinergic receptor antagonists need to be assessed. Functional effects in addition to the location of these purinoceptors need to be investigated further.
- (iv) As with most animal experiments there is difficulty in translating the work into human models. With the work on the isolated guinea pig bladder an attempt has been made to relate structure to function. This working principle needs to be

repeated in other animal models, so that the effects of both unique and common structures can be assessed. Following which the structures and workings of the human bladder can be speculated.

- (v) The work of Hawthorn et al, 2000 in suggesting the production of a readily diffusible 'inhibitory factor' derived from the urothelium could be assessed further in this organ preparation. It would be interesting to see whether volume distension of one isolated bladder in close proximity to another induces an 'inhibitory phase', and whether this would occur in a bladder in iso-volumetric conditions. If so, the location of the mechanisms involved may allow the development of a focused therapeutic target that could reduce non-micturition activity; and therefore it's associated afferent discharge.

- (vi) Additionally, more focus needs to be brought upon high gain urodynamics performed at physiological fill rates in order to catalogue the characteristics of non-micturition activity in both the normal and diseased states.

REFERENCES

Chapter Six

Abrams P, Cardozo L, Fall M, Griffiths D, Rosier P, Ulmsten U. The standardisation of terminology of lower urinary tract function: report from the Standardisation Subcommittee of the International Continence Society. *Neurourol Urodyn* 2002; 21: 167-78.

Abrams P, Kaplan S, Millard R. Safety of Tolterodine in men with outlet obstruction and symptomatic detrusor overactivity. *Eur Urol* 2002; 1: 132 [abstract 520].

Andersson KE, Arner A. Urinary bladder contraction and relaxation: physiology and pathophysiology. *Physiol Rev* 2004; 84: 935-86.

Andersson KE, Wein AJ. Pharmacology of the lower urinary tract: basis for current and future treatments of urinary incontinence. *Pharmacol Rev* 2004; 56(4): 581-631.

Andersson K-E, Yoshida M. Antimuscarinics and the Over Active Detrusor – Which is the main mechanism of action? *European Urology* 2003; 43: 1-5.

Andersson K-E. Bladder activation: Afferent mechanism. *Urology* 2002; 59: 43-50.

Athanasopoulos A, Ghyftopoulos K, Giannitsas K, Fisfis J, Perimenis P, Barbalias G. Combination treatment with an [alpha]-blocker plus an antimuscarinic for bladder outlet obstruction: A prospective randomised, controlled study. *J Urol* 2003; 169(6): 2253-2256.

Barlow RB, Shepherd MK. A further search for selective antagonists at M₂- muscarinic receptors. *Br J Pharmacol* 1986; 89: 837.

Barlow RB, Weston-Smith P. The relative potencies of some agonists at M₂ muscarinic receptors in guinea pig ileum, atria and bronchi. *Br J Pharmacol* 1985; 85: 437.

Bayliss M, Wu C, Newgreen D, Mundy AR, Fry CH. A quantitative study of atropine resistant contractile responses in human detrusor smooth muscle, from stable, unstable and obstructed bladders. *J Urol* 1999; 168: 2040-5.

Beckel JM, Kanai A, Lee SJ, de Groat WC, Birder LA. Expression of functional nicotinic acetylcholine receptors in rat urinary bladder epithelial cells. *Am J Physiol Renal Physiol* 2006; 290(1): F103-10.

Billard W, Binch H 3rd, Crosby G, McQuade RD. Identification of the primary muscarinic autoreceptor subtype in rat striatum as M₂ through a correlation of in vivo microdialysis and in vitro receptor binding data. *J Pharmacol Exp Ther* 1995; 273: 273.

Birder LA, Nealen ML, Kiss S, de Groat WC, Caterina MJ, Wang E, Apodaca G, Kanai AJ. Beta-adrenoceptor agonists stimulate endothelial nitric oxide synthase in rat urinary bladder urothelial cells. *J Neurosci* 2002; 22(18): 8063-8070.

Birder LA, Kanai AJ, de Groat WC, Kiss S, Nealen ML, Burke NE, Dineley KE, Watkins S, Reynolds IJ, Caterina MJ. Vanilloid receptor expression suggests a sensory

role for urinary bladder epithelial cells. *Proc Natl Acad Sci U S A* 2001; 98(23): 13396-13401.

Birder LA, Apodaca G, De Groat WC & Kanai AJ. Adrenergic- and capsaicin- evoked nitric oxide release from urothelium and afferent nerves in urinary bladder. *Am J Physiol* 1998; 275: F226-229.

Boyer JL, Mohanram A, Camaioni E, Jacobson KA, Harden TK. Competitive and selective antagonism of P2Y1 receptors by N6 – methyl 2' – deoxyadenosine 3', 5' – biphosphate. *Br J Pharmacol* 1998; 124(1): 1.

Brading AF, Turner WH. The unstable bladder: towards a common mechanism. *Br J Urol* 1994; 73: 3-8.

Bristow SE, Neal DE. Ambulatory urodynamics. *B J Urol* 1996; 77: 333-338.

Burnstock G, Cusack NJ, Hills JM, MacKenzie I, Meghji P. Studies on the stereoselectivity of the P₂ – purinoreceptor. *Br J Pharmacol* 1983; 79(4): 907-13.

Cardozo L, Chapple CR, Tooze-Hobson P, Grosse-Freese M, Schäfer M, Bulitta M, Lehmacher W, Strosser W, Ballering – Bruhl B. Efficacy of Trosipium Chloride in patients with detrusor instability: a placebo-controlled, randomised, double blind, multicentre clinical trial. *BJU Int* 2000; 85(6): 644-659.

Caulfield MP, Birdsall NJM. International Union of Pharmacology : XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol Rev* 1998; 50: 279-290.

Chapple C, Khullar V, Gabriel Z, Dooley JA. The effects of antimuscarinic treatments in Overactive Bladder: A systematic review and meta-analysis. *Eur Urol* 2005; 48(1): 5-26.

Chess-Williams R. Potential therapeutic targets for the treatment of detrusor overactivity. *Expert Opin Ther Targets* 2004; 8: 95-106.

Chess-Williams R, Chapple CR, Yamanishi T, Yasuda K, Sellers DJ. The minor population of M3 receptors mediate contraction of human detrusor muscle in vitro. *J Auton Pharmacol* 2001; 21: 243-8.

Cook SP, McCleskey EW. ATP, pain and a full bladder. *Nature* 2000; 407(6807): 951-2.

Cusack NJ, Hourani SM. Specific but non-competitive inhibition by 2-alkylthio analogues of adenosine 5'- monophosphate and adenosine 5'- triphosphate of human platelet aggregation induced by adenosine 5'- diphosphate. *Br J Pharmacol* 1982; 75(2): 397-400.

Dammer S, Niebel B, Czeche S, Nickel P, Ardanuy U, Schmalzing G, Rettinger J, Mutschler E, Lambrecht G. NF279: a novel potent and selective antagonist of P2X receptor mediated responses. *Eur J Pharmacol* 1998; 350(1): R5-6.

Davidson RA, McCloskey KD. Morphology and localization of interstitial cells in the guinea pig bladder: structural relationships with smooth muscle and neurons. *J Urol* 2005; 173(4):1385-1390.

Denny-Brown D, Roberston EG. On the physiology of micturition. *Brain* 1933: 56, 149.

Digesu G, Khullar V, Cardozo L, Salvatore S. Overactive bladder symptoms: do we need urodynamics? *Neurourol Urodyn*. 2003; 22: 105 – 108.

Drake MJ, Harvey IJ, Gillespie JI, van Duyl WA. Localised contractions in the normal human bladder and in urinary urgency. *BJU Int* 2005: 95; 1002-1005.

Drake MJ, Harvey IJ, Gillespie JI. Autonomous activity in the isolated guinea pig bladder. *Exp Physiol* 2003a; 88(1): 19-30.

Drake MJ, Hedlund P, Harvey IJ, Pandita RK, Andersson K-E, Gillespie JI. Partial outlet obstruction enhances modular autonomous activity in the isolated rat bladder. *J Urol* 2003b; 170(1): 276-279.

- Drake MJ, Harvey IJ & Gillespie JI. Agonist and nerve induced phasic activity in the isolated whole bladder of the guinea pig: evidence for two types of bladder activity. *Exp Physiol* 2003c; 88(3): 343-357.
- Eglen RM, Hegde SS, Watson N. Muscarinic receptor subtypes and smooth muscle function. *Pharmacol Rev* 1996; 48(4): 531-65.
- Ferguson DR. Urothelial function. *BJU Int* 1999; 84: 235-242.
- Ferguson DR, Kennedy I, Burton TJ. ATP is released from rabbit urinary bladder epithelial cells by hydrostatic pressure changes – a possible sensory mechanism? *J Physiol* 1997; 505: 503-11.
- Fetscher C, Fleischman M, Schmidt M, Kroke S, Michel MC. M(3) muscarinic receptors mediate contraction of human urinary bladder. *Br J Pharmacol* 2002; 136: 641-4.
- Finney S, Andersson KE, Gillespie JI, Stewart LH. Antimuscarinic drugs in detrusor overactivity and the overactive bladder syndrome: motor or sensory actions? *BJU Int* 2006; 90(3): 503-07.
- Fry CH, Skennerton D, Wood D, Wu C. The cellular basis of contraction in human detrusor smooth muscle from patients with stable and unstable bladders. *J Urol* 2002; 59: 3-12.

Fryer AD, MacLagan J. Pancuronium and gallamine are antagonists for pre- and post-junctional muscarinic receptors in the guinea pig lung. *Naunyn-Schmeid. Arch. Pharmacol* 1987; 335(4): 367-71.

Gabella G. Intramural neurons in the urinary bladder of the guinea pig. *Cell Tissue Res* 1990 Aug; 261(2): 231-7.

Giannitsas K, Perimenis P, Athanasopoulos A, Gryftopoulos K, Nikiforidis G, Barbalias G. Comparison of the efficacy of Tolterodine and Oxybutynin in different urodynamic severity grades of idiopathic detrusor overactivity. *Eur Urol* 2004; 46: 776-783.

Gillespie JJ, Markerink-van Ittersum M, de Vente J. Interstitial cells and cholinergic signalling in the outer muscle layers of the guinea pig bladder. *BJU Int* 2006; 97(2): 379-85.

Gillespie JJ, Markerink-van Ittersum M, de Vente J. Sensory collaterals, intra-mural ganglia and motor nerves in the guinea pig bladder: evidence for intramural neural circuits *Cell and Tissue Res* 2006a; 325(1): 33-45.

Gillespie JJ, Markerink-van Ittersum M, de Vente J. cGMP generating cells in the bladder wall: identification of distinct networks of interstitial cells. *BJU Int* 2005; 94: 1114-1124.

Gillespie JJ. Inhibitory actions of calcitonin gene related peptide and capsaicin: evidence for local axonal reflexes in the bladder wall. *BJU Int* 2005a; 95(1): 149-56.

Gillespie JJ. A developing view of the origins of urgency: the importance of animal models. *BJU Int* 2005b; 96: 22-28.

Gillespie JJ. The Autonomous Bladder: A View of the Origin of Bladder Overactivity. *BJU Int* 2004; 93: 478-483.

Gillespie JJ. Modulation of autonomous contractile activity in the isolated whole bladder of the guinea pig. *BJU Int* 2004a; 93(3); 393-400.

Gillespie JJ, Drake MJ. The actions of sodium nitroprusside and the phosphodiesterase inhibitor dipyridamole on phasic activity in the isolated guinea pig bladder. *BJU Int* 2004b; 93(6): 851-8.

Gillespie JJ. Noradrenaline inhibits autonomous activity in the isolated guinea pig bladder. *BJU Int* 2004c; 93(3): 401-9.

Gillespie JJ. PDE Linked Inhibition of Non-micturition Activity in the Isolated Bladder. *BJU Int* 2004d; 93: 1325-1332.

Gillespie JJ, Markerink-van Ittersum M, de Vente J. cGMP generating cells in the bladder wall : identification of distinct networks of interstitial cells. *BJU Int*. 2004 ; 94(7) : 1114-1124.

Gjone R. Peripheral autonomic influence on the motility of the urinary bladder in the cat. I. Rhythmic contractions. *Acta Physiol Scand* 1965; 65: 370-377.

Goessl C, Sauter T, Michael T, Bergé B, Staehler M, Miller K. Efficacy and tolerability of Tolterodine in children with detrusor hyperreflexia. *Urol* 2000; 55(3): 414-418.

Goode P, Burgio KL, Locher JL, Umlauf MG, Llyod KL, Roth DL. Urodynamic changes associated with behavioural and drug treatment of urge incontinence in older women. *J Am Geriatr Soc* 2002; 50(5): 808-816.

Griffiths D, Harrison G, Moore K, M^cCracken P. Long term changes in urodynamic studies of voiding in the elderly. *Urol Res* 1994; 22: 235-238.

de Groat WC. The urothelium in overactive bladder: passive bystander or active participant. *Urology* 2004; 64(6 suppl 1): 7-11.

de Groat WC. A neurologic basis for the overactive bladder. *Urology* 1997; 50 (suppl 6A): 36-52s.

Guarneri L, Ibba M, Angelico P, Testa R. Effects of Oxybutynin, Terodiline, and Nifedipine on the cystometrogram in conscious rats with infravesical outflow obstruction. *Pharm Res* 1991; 24: 263-272.

- Hannappel J, Lutzeyer W. Pacemaker localisation in the renal pelvis of the unicalyceal kidney. In vitro study in the rabbit. *Eur Urol* 1978; 4(3): 192-4.
- Hammer R, Giraldo E, Schiavi GB, Monferini E, Ladinski H. Binding profile of a novel cardioselective muscarinic receptor antagonist, AF-DX 116, to membranes of peripheral tissues and brain in the rat. *Life Sci* 1986; 38: 1653.
- Hashimoto K, Ohnishi N, Esa A, Sugiyama T, Park Y, Kurita T. Clinical efficacy of Oxybutynin on sensory urgency as compared with that on motor urgency. *Urol Int.* 1999; 62(1): 12-6.
- Hashitani H, Yanai Y, Suzuki H. Role of interstitial cells and gap junctions in the transmission of spontaneous Ca^{2+} signals in detrusor smooth muscles of the guinea-pig urinary bladder. *J Physiol* 2004; 559: 567-581.
- Hawthorn MH, Chapple CR, Cock M, Chess-Williams R. Urothelium derived inhibitory factor(s) influences on detrusor muscle contractility in vitro. *Br J Pharmacol* 2000; 129: 416-9.
- Hegde SS, Eglen RM. Muscarinic receptor subtypes modulating smooth muscle contractility in the urinary bladder. *Life Sci* 1999; 64: 419-28.
- Hegde SS, Choppin A, Bonhaus D, Briaud S, Loeb M, Moy TM, Loury D, Eglen RM. Functional role of M2 and M3 muscarinic receptors in the urinary bladder of rats in vitro and in vivo. *Br J Pharmacol* 1997; 120(8): 1409-18.

- Hertle L, Nawrath H. Calcium channel blockade in smooth muscle of the human upper tract. I. Effects on depolarization-induced activation. *J Urol* 1984; 132(6): 1265-9.
- Heslington K and Hilton P. Ambulatory monitoring and conventional cystometry in asymptomatic female volunteers. *Br J Obst Gynaecol.* 1996; 103: 434 - 441.
- Hou VY, Hirshman CA, Emala CW. Neuromuscular relaxants as antagonists for M2 and M3 muscarinic receptors. *Anesthesiology* 1998; 88(3): 744-50.
- Hu T-W, Wagner TH, Bentkover JD, leBlanc K, Piancentini A, Stewart WF, Covey R, Zhou SZ, Hunt TL. Estimated economic costs of overactive bladder in the united states. *Urology* 2004; 63(3): 461-5.
- Huizinga JD, Thuneberg L, Vanderwinden JM, Rumessen J. Interstitial cells of Cajal as targets for pharmacological intervention in gastrointestinal disorders. *Trend Pharmacol Sci* 1997; 18: 393-402.
- Huizinga JD, Thuneberg L, Klüppel M, Malysz J, Mikkelesen H, Bernstein A. W/kit gene required for interstitial cells of Cajal and for intestinal pacemaker activity. *Nature* 1995; 373: 347-349.
- Iggo A. Tension receptors in the stomach and urinary bladder. *J Physiol* 1955: 128: 593-607.

Inoue R, Brading AF. The properties of the ATP-induced depolarisation and current in single cells isolated from the guinea pig urinary bladder. *Br J Pharmac* 1990; 100: 619-25.

Inoue R, Brading AF. Human, pig and guinea pig bladder smooth muscle cells generate similar inward currents in response to purinoceptor activation. *Br J Pharmacol*. 1991; 103(4): 1840 - 1841.

Jonas U, Höffner K, Madersbacher H, Holmdahl TH. Efficacy and safety of two doses of Tolterodine versus placebo in patients with detrusor overactivity and symptoms of frequency, urge incontinence, and urgency: urodynamic evaluation. *World J Urol* 1997; 15: 144-151.

Khakh BS, Burnstock G, Kennedy C, King BF, North RA, Seguela P, Voigt M, Humphrey PP. International union of Pharmacology. XXIV. Current status of the nomenclature and properties of P2X receptors and their subunits. *Pharmacol Rev* 2001; 53(1): 107-18.

Kim Y, Yoshimura N, Masuda H, de Miguel F, Chancellor MB. Antimuscarinic agents exhibit local inhibitory effects on muscarinic receptors in bladder afferent pathways. *Urology* 2005; 65(2): 238-42.

Klapperstuck H, Buttner C, Nickel P, Schmalzing G, Lambrecht G, Markwardt F. Antagonism by the suramin analogue NF279 on human P2X(1) and P2X(7) receptors. *Eur J Pharmacol* 2000; 387(3): 245-52.

Klemm MF, Exintaris B, Lang RJ. Identification of the cells underlying pacemaker activity in the guinea pig upper urinary tract. *J Physiol*. 1999; 519 Pt 3: 867-84.

Klevmark B. Volume threshold for micturition. Influence of filling rate on sensory and motor bladder function. *Scand J Urol Nephrol* 2002; Suppl 210; 6 -10.

Klevmark B. Motility of the urinary bladder in cats during filling at physiological rates. III. Spontaneous rhythmic bladder contractions in the conscious and anesthetised animal. Influence of distension and innervation. *Scand J Urol Nephrol* 1980; 14(3): 1219-224.

Kobayashi S, Chowdhury JU, Tukuno H, Nahar S, Iino S. A smooth muscle nodule producing 10-12 cycles/ min regular contractions at the mesenteric border of the pacemaker area in the guinea pig colon. *Archiv Histol Cytol* 1996; 59: 159-168.

Kobayashi S, Torihashi S, Iino S, Pang YW, Chowdhury JU, Tomita T. The inner sublayer of the circular muscle coat in the canine proximal colon: origins of spontaneous electrical and mechanical activity. *Archiv Histol Cytol* 1995; 58: 45-63.

Lagou M, Drake M, Gillespie JJ. Volume induced effects on the isolated bladder: a possible reflex. *BJU Int* 2004; 94(9): 1356-65.

Lee HY, Bardini M, Burnstock G. Distribution of P2X receptors in the urinary bladder and the ureter of the rat. *J Urol* 2000; 163: 2002-7.

Lennon GM, Ryan PC, Fitzpatrick JM. The ureter in vitro: normal motility and response to urinary pathogens. *Br J Urol* 1993a; 72(3): 284-90.

Lennon GM, Ryan PC, Fitzpatrick JM. Recovery of ureteric motility following complete and partial ureteric obstruction. *Br J Urol* 1993b; 72 (5 Pt 2): 702-7.

Lowenick CV, Krampfl K, Schneck H, Kochs E, Bufler J. Open channel and competitive block of nicotinic receptors by pancuronium and atracurium. *Eur J Pharmacol* 2001; 413(1): 31-5.

Lambrecht G, Friebe T, Grimm U, Windscheif U, Bungardt E, Hildebrandt C, Braumert HG, Spatz-Kumbel G, Mutschler E. PPADS, a novel functionally selective antagonist of P2 purinoceptor – mediated responses. *Eur J Pharmacol* 1992; 217(2-3): 217-9.

Madersbacher H, Halaska M, Voigt R, Alloussi S, Höffner K. A Placebo controlled, multicentre study comparing the tolerability and efficacy of Propiverine and Oxybutynin in patients with urgency and urge incontinence. *BJU Int* 1999; 84: 646-651.

Madersbacher H, Stöhrer M, Richter R, Burgdörfer H, Hachen HJ, Mürtz G. Trospium Chloride versus Oxybutynin: a randomised, double blind, multicentre trial in the treatment of detrusor hyper-reflexia. *BJU* 1995; 75: 452-456.

Maestrone E, Magnelli V, Nobile M, Usai C. Extracellular pancuronium affects sodium current in chick embryo sensory neurones. *Br J Pharmacol* 1994; 111(1): 283-7.

Maggi CA. The dual function of capsaicin sensory nerves in the bladder and urethra. *Ciba Found Symp* 1990; 151: 77-83.

Maggi CA, Meli A. The sensory-efferent function of capsaicin sensitive sensory neurons. *General Pharmacol* 1988; 19: 1-43.

Maggi CA, Meli A. The role of neuropeptides in the regulation of the micturition reflex. *J Auton Pharmacol* 1986; 6: 133-62.

Mansfield KJ, Liu L, Mitchelson FJ, Moore KH, Millard RJ, Burcher E. Muscarinic receptor subtypes in human bladder detrusor and mucosa, studied by radioligand binding and quantitative competitive RT-PCR: changes in ageing. *Br J Pharmacol*. 2005; 144(8): 1089-99.

McCloskey K D, Gurney A M. *kit*-positive cells in the guinea pig bladder. *J Urol* 2002; 168; 832-836.

McLaren GJ, Lambrecht G, Mutschler E, Baumert HG, Sneddon P, Kennedy C. Investigation of the actions of PPADS, a novel P2X – purinoceptor antagonist, in the guinea pig isolated vas deferens. *Br J Pharmacol* 1994; 111(3): 913-7.

McMurray G, Dass N, Brading AF. Purinoceptor subtypes mediating contraction and relaxation of marmoset urinary bladder smooth muscle. *Br J Pharmac* 1998; 123: 1579-86.

Michel AD, Stefanich E, Whiting RL. Direct labelling of rat M₃-muscarinic receptors by [³H]4-DAMP. *Eur J Pharmacol* 1989; 166(3): 459-66.

Milsom I, Abrams P, Cardozo L, Roberts RG, Thuroff J, Wein AJ. How widespread are the symptoms of an overactive bladder and how are they managed? A population based prevalence study. *BJU Int* 2001; 87: 760-6.

Mitsui T, Kakizaki H, Matsuura S, Ameda K, Yoshioka M, Koyanagi T. Afferent fibers of the hypogastric nerves are involved in the facilitating effects of chemical bladder irritation in rats. *J Neurophysiol* 2001 Nov; 86(5): 2276-84.

Morro S, Guo D, Camaioni E, Bayer JL, Harden TK, Jacobson KA. Human P2Y₁ receptor molecular modelling and site directed mutagenesis as tools to identify agonist and antagonist recognition sites. *J Med Chem* 1998; 41: 1456.

Morrison JF. The activation of bladder wall afferent nerves. *Exp Physiol*. 1998; 82: 902-905.

Moser U, Lambrecht G, Wagner M, Wess J, Mutschler E. Structure-activity relationships of new analogues of arecaidine propargyl ester at muscarinic M₁ and M₂ receptor subtypes. *Br J Pharmacol* 1989; 96(2): 319-24.

Mosso MA, Pellacani P. Sur le fonctions de la vessie. *Arch Ital Biol* 1882; 1: 291-324.

Nandanan E, Jang SY, Moro S, Kim HO, Siddiqui MA, Russ P, Marquez VE, Bussan R, Herdewijn P, Harden TK, Boyer JL, Jacobson KA. Synthesis, biological activity, and molecular modelling of ribose -modified deoxyadenosine biphosphate analogues as P2Y1 receptor ligands. *J Med Chem* 2000; 43(5): 829-42.

Nelson CP, Gupta P, Napier CM, Nahorski SR, Challiss RA. Functional selectivity of muscarinic receptor antagonists for inhibition of M3-mediated phosphoinositide responses in guinea pig urinary bladder and submandibular salivary gland. *J Pharmacol Exp Ther* 2004; 310(3): 1255 – 65.

Palae S, Artibani W, Ostardo E, Trist DG, Pietra C. Evidence for neurotransmission in human urinary bladder affected by interstitial cystitis. *J Urol* 1993; 150: 2007-12.

Plum F, Colfelt RH. The genesis of vesical rhythmicity. *AMA Arch of Neurol* 1960; 2: 487 - 496.

Rettinger J, Schmalzing G, Damer S, Muller G, Nickel P, Lambrecht G. The suramin analogue NF279 is a novel and potent antagonist selective for the P2X(1) receptor. *Neuropharmacology* 2000; 39(11): 2044-53.

Robertson A, Griffiths C, Ramsden P and Neal D: Bladder function in healthy volunteers: ambulatory monitoring and conventional urodynamic studies. *BJU* 1994; 73: 242 – 249.

Rong W, Spyer KM, Burnstock G. Activation and sensitisation of low and high threshold afferent fibres mediated by P2X receptors in the mouse urinary bladder. *J Physiol* 2002; 541(Pt 2): 591-600.

Rosario DJ, Cutinha PE, Chapple CR, Milroy E. The effects of single dose Darifenacin on cystometric parameters and salivary flow in patients with urge incontinence secondary to detrusor instability. *Eur Urol* 1996; 30(Suppl. 2): 250 (Abstract 896).

Ryan PC, Lennon GM, McLean PA, Fitzpatrick JM. The effects of acute and chronic JJ stent placement on upper urinary tract motility and calculus transit. *Br J Urol* 1994; 74(4): 434-9.

Sanders KM, Ördög T, Koh SD, Torihashi S, Ward SM. Development and plasticity of interstitial cells of Cajal. *Neurogastroenterol Motil* 1999;11: 311-338.

Sherrington C S. Notes on the arrangement of some motor fibres in the lumbosacral plexus. *J Physiol* 1892; 13: 621-772.

Smet PJ, Moore KH, Jonavicius J. Distribution and co-localisation of calcitonin gene related peptide, tachykinins, and vasoactive intestinal peptide in normal and idiopathic unstable human urinary bladder. *Lab Invest* 1997 Jul; 77(1): 37-49.

Smet PJ, Jonavicius J, Marshall VR, de Vente J. Distribution of nitric oxide synthase immunoreactive nerves and identification of the cellular targets of nitric oxide in guinea pig and human urinary bladder by cGMP immunohistochemistry. *Neuroscience* 1996; 71: 337-48.

Somogyi GT, de Groat WC. Function, signal transduction mechanisms and plasticity of presynaptic muscarinic receptors in the urinary bladder. *Life Sci* 1999; 64(6-7): 411–8.

Stahl MMS, Ekström B, Sparf B, Mattiasson A, Andersson KE. Urodynamic and other effects of Tolterodine: A novel antimuscarinic drug for the treatment of detrusor overactivity. *Neurourol Urodyn* 1995; 14: 647-655.

Starling EH. Elements of Human Physiology, 7th edn. London. Churchill, 1905.

Stewart WF, Herzog A, Wein A, Abrams P, Payne C, Corey R, Hunt T, NOBLE program research team. Prevalence and impact of overactive bladder in the US: results from the NOBLE program. *Neurol Urodyn* 2001; 20(4): 406(abstract 24).

Stöhrer M, Bauer P, Giannetti BM, Richter R, Burgdörfer H, Mürtz G. Effect of Trospium Chloride on urodynamic parameters in patients with detrusor hyperreflexia due to spinal cord injuries. *Urol Int* 1991; 47: 138-143.

Streng T, Hedlund P, Talo A, Andersson KE, Gillespie JJ. Phasic non-micturition contractions in the bladder of the anaesthetised and awake rat. *BJU Int* 2006; 97(5): 1094-1101.

Sui GP, Wu C, Fry CH. Characterisation of the purinergic receptor subtypes on guinea pig suburothelial myofibroblasts. *BJU Int* 2006; 97(6): 1327-31.

Suzuki M, Ohtake A, Yoshino T, Yuyama H, Hayashi A et al. Effects of Solifenacin Succinate (YM905) on detrusor overactivity in conscious cerebral infarcted rats. *Eur J Pharmacol* 2005; 512(1): 61-6.

Takeda H, Yamazaki Y, Igawa Y, Kaidoh K, Akahane S, Miyata H, Nishizawa O, Akahane M, Andersson KE. Effects of beta(3)-adrenoceptor stimulation on prostaglandin E(2)-induced bladder hyperactivity and on the cardiovascular system in conscious rats. *Neurourol Urodyn* 2002; 21(6): 558-65.

Thüroff JW, Bunke B, Ebner A, Faber P, Stöckle M. Randomised, double blind, multicentre trial on treatment of frequency, urgency and incontinence related to detrusor hyperactivity: Oxybutynin versus Propantheline versus placebo. *J Urol* 1991; 145: 813-817.

Thulesius O, Angelo-Khattar M. The effect of indomethacin on the motility of isolated sheep ureters. *Acta Pharmacol Toxicol (Copenh)*. 1985; 56(4): 298-301.

Tonini M, Rizzi CA, Perucca E, de Ponti, D'Angelo L, Del Vecchio A, Crema A. Depressant action of Oxybutynin on the contractility of intestinal and urinary tract smooth muscle. *J Pharm Pharmacol* 1987; 39(2): 103-7.

Tyagi S, Tyagi P, Van-le S, Yoshimura N, Chancellor M, de Miguel F. Qualitative and quantitative expression profile of muscarinic receptors in human urothelium and detrusor. *J Urol* 2006; 176(4): 1673-1678.

Wagg A, Malone-Lee J. Pressure-flow variables in patients treated with Tolterodine for detrusor overactivity. *BJU Int* 2003; 92: 969-971.

Waldeck K, Larsson B, Andersson KE. Comparison of Oxybutynin and its active metabolite , N-desethyl-oxybutynin, in the human detrusor and parotid gland. *J Urol* 1997; 157: 1093-1097.

Wang P, Luthin GR, Ruggieri MR. Muscarinic acetylcholine receptor subtypes mediating urinary bladder contractility and coupling to GTP binding proteins. *Pharm* 1995; 273(2): 959 - 966.

Wu C, Sui GP, Fry CH. Purinergic regulation of guinea pig suburothelial myofibroblasts. *J Physiol* 2004; 559: 231-243.

Van Os-Bossagh P, Kosterman LM, Hop WC, Westerhof BE, de Bakker JV, Drogendijk AC, van Duyl WA. Micromotions of bladder wall in chronic pelvic pain (CPP): a pilot study. *Int Urogynecol J Pelvic Floor Dysfunct* 2001; 12(2); 89-96.

Vaughan CW, Satchell PM. Urine storage mechanisms. *Prog Neurobiol* 1995; 46(2-3): 215-237.

Vaughan CW, Satchell PM. Role of sympathetic innervation in the feline continence process under natural filling conditions. *J Neurophysiol* 1992; 68(5): 1842-1849.

- Yamanishi T, Chapple CR, Yasuda K, Chess-Williams R. The role of M2 muscarinic receptor subtypes in mediating contraction of the pig bladder base after cyclic adenosine monophosphate elevation and/ or selective M3 inactivation. *J Urol* 2002; 167: 397-401.
- Yamanishi T, Chapple CR, Chess-Williams R. Which muscarinic receptor is important in the bladder? *World J Urol* 2001; 19: 299-306.
- Yokoyama O, Yusup A, Miwa Y, Oyama N, Aoki Y, Akino H. Effects of tolterodine on an overactive bladder depends on suppression of C-fiber bladder afferent activity in rats. *J Urol*. 2005; 174(5): 2032-6.
- Yono M, Yoshida M, Wada Y, Kikukawa H, Takahashi W, Inadome A, Seshita H, Ueda S. Pharmacological effects of Tolterodine on human isolated urinary bladder. *Eur J Pharmacol* 1999; 368(2-3): 223-230.
- Yoshida M, Miyamae K, Iwashita H, Otani M, Inadome A. Management of detrusor dysfunction in the elderly: changes in acetylcholine and adenosine triphosphate release during aging. *Urology*. 2004; 63(3 suppl 1): 17-23.
- Zhou Y, Ling EA. Co-localisation of nitric oxide synthase and some other neurotransmitters in the intramural ganglia of the guinea pig bladder. *J Comp Neurol* 1998 May 18; 394(4): 496 -505.

Ziganshin AU, Hoyle CH, Bo X, Lambrecht G, Mutschler E, Baumert HG, Burnstock G. Selective antagonism by PPADS at P2X – purinoceptors in rabbit isolated blood vessels. *Br J Pharmacol* 1994; 111(3): 923-9.

PUBLISHED PAPERS

CHAPTER SEVEN

The three enclosed papers were published in the British Journal of Urology International during the years 2006, 2007 and 2008. Permission for their inclusion in this thesis has been granted from both the publisher and the co-authors.

Antimuscarinic drugs in detrusor overactivity and the overactive bladder syndrome: motor or sensory actions?

STEVEN M. FINNEY, KARL-ERIK ANDERSSON*, JAMES I. GILLESPIE† and LAURENCE H. STEWART

Western General Hospital, Edinburgh, UK, *Department of Clinical and Experimental, Pharmacology, Lund University Hospital, Sweden, and †The Urophysiology Research Group, School of Surgical and Reproductive Sciences, The Medical School, The University, Newcastle upon Tyne, UK

Accepted for publication 14 March 2006

Antimuscarinic drugs are generally thought to exert their therapeutic action on detrusor overactivity by reducing the ability of the detrusor muscle to contract. We review currently available published data to establish whether there is any evidence to support this contention. Using a PubMed data search, only 14 original articles (including two abstracts) were found that contained cystometric data for both filling and voiding phases and where the actions of antimuscarinic drugs have been reported in detail. These articles were separated into three groups dealing with neuropathic patients (three papers), patients with idiopathic overactive bladder (four papers)

and a group whose aetiology was unclear (seven papers). Variables relating to bladder function during the filling phase (time of first desire to void, time to first unstable contraction, and bladder capacity) were identified. Similarly, variables relating to voiding were identified and compared (e.g. maximum detrusor pressure and detrusor pressure at maximum flow rate). The antimuscarinic drugs have a clearly significant effect on sensations of urge, time to first sensation to void, maximum bladder capacity, decrease in voiding frequency and reduction in incontinence episodes. However, only one article (studying neuropaths) reported a significant reduction of the

variables associated with detrusor contraction. The remaining four studies (idiopathic/not stated), reported no change in bladder contractility with antimuscarinic drugs. Thus the available data do not support the conclusion that antimuscarinic drugs at doses used in current clinical practice exert their therapeutic action by inhibiting detrusor contractility, but they suggest effects on variables associated with sensation.

KEYWORDS

anticholinergic drugs, bladder overactivity, urinary urge

INTRODUCTION

According to current ICS terminology detrusor overactivity (DO) is defined as 'a urodynamic observation characterized by involuntary detrusor contractions during the filling phase which may be spontaneous or provoked'. The overactive bladder syndrome (OAB) is a symptom complex characterized by urgency, with or without urgency incontinence, usually accompanied by frequency and nocturia [1]. It is a common condition affecting 17% of the populations of Europe and the USA [2,3]. Most patients with OAB have urodynamically confirmed DO, and the vast majority of patients with DO have symptoms of OAB [4].

The symptoms of DO/OAB have been generally considered to be due to abnormal bladder contractions occurring inappropriately during the filling phase. On this assumption the mainstay of treatment for OAB has been antimuscarinic medication, with the contention that they will reduce the contractility of the detrusor muscle. The logical argument was that by preventing these abnormal contractions this would

relieve the symptoms. There is no doubt that the antimuscarinic drugs currently used in clinical practice (oxybutynin, tolterodine, trospium, solifenacin and darifenacin) are effective in reducing the symptoms of OAB. Also, there is no doubt that at high doses antimuscarinics can reduce the amplitude of detrusor contraction. Indeed, patients given an excess of antimuscarinic drugs retain urine because the bladder cannot contract. However, what is the evidence that the therapeutic effect of these drugs is to reduce bladder contractility?

Our aim in this review is to analyse previously reported data related to therapeutic doses of antimuscarinics in current routine use on bladder contractility and sensory variables.

PRECLINICAL STUDIES

It is generally accepted that detrusor contraction in humans is mainly mediated by acetylcholine released from cholinergic nerves, with acetylcholine stimulating muscarinic receptors on the detrusor myocytes. Although all muscarinic receptor subtypes have been detected in the human

detrusor, the M_2 and M_3 subtypes predominate. Although M_2 receptors might predominate in number over M_3 receptors, it is the M_3 receptors that are mainly responsible for the normal micturition contraction [5]. Muscarinic receptors are also found on bladder urothelial cells and on structures in the suburothelium (interstitial cells, nerves) [6]. Therefore antimuscarinics block, more or less selectively, muscarinic receptors not just on the detrusor muscle, where they decrease the ability of the bladder to contract. However, clinically, antimuscarinics act mainly during the storage phase, decreasing urgency and increasing bladder capacity. During this phase it is generally considered that there is no activity in the parasympathetic nerves. Furthermore, antimuscarinics are usually competitive antagonists. This implies that when there is a massive release of acetylcholine, as during micturition, the effects of the drugs should be decreased, otherwise the reduced ability of the detrusor to contract would eventually lead to urinary retention. The question is whether there are other effects of antimuscarinics that can contribute to their beneficial effects in the treatment of OAB.

The urothelium/suburothelium has attracted considerable interest as a target for antimuscarinics. Yoshida *et al.* [7] found that there is a basal acetylcholine release in isolated human bladder tissue. This release was suggested to be of non-neuronal origin and, at least partly, generated by the urothelium. Intravesical administration of muscarinic receptor agonists can generate DO, which can be inhibited by antimuscarinics [8] and it was suggested that such an effect was mediated via afferent nerves in the suburothelium. Further supporting an effect of antimuscarinics on afferent nerves, Yokoyama *et al.* [9] found that low i.v. doses of tolterodine significantly increased bladder capacity in vehicle-treated rats with DO caused by cerebral infarction, but had no effects on bladder capacity in rats treated with resiniferatoxin. Intravesical administration of tolterodine significantly increased bladder capacity in control rats with cerebral infarction, but had no effect on bladder capacity in resiniferatoxin-treated rats. Yokoyama *et al.* therefore suggested that at low doses tolterodine has an inhibitory effect on C-fibre bladder afferent nerves, thereby improving bladder capacity during the storage phase.

EVIDENCE FROM STUDIES ON PATIENTS

Articles containing cystometric data for patients treated with antimuscarinics, currently used in routine clinical practice, were identified through a standard Medline search. Generic and brand names were cross-referenced with terms used to describe urodynamic assessments, in addition to filling/voiding variables. Few studies were identified, which is surprising given the prevalence of OAB and the global usage of antimuscarinics.

There are only 14 original articles (including two abstracts) [10–23] which contain cystometric data for both filling and voiding phases in patients with OAB before and after treatment. In these articles there is much variation in the way cystometric variables are reported, the numbers of patients involved and methods of analysis. These factors make a significant formal statistical analysis difficult. In addition, the changing definition of an 'overactive contraction' during cystometry, from those restricted to >15 cmH₂O to all clinically relevant involuntary rises in detrusor pressure (Pdet), makes a cumulative comparison inappropriate, as a further

TABLE 1 Details of the papers reporting results in group I

Variable	Madersbacher <i>et al.</i> [10]	Madersbacher <i>et al.</i> [10]	Goesl <i>et al.</i> [11]	Stöhrer <i>et al.</i> [12]
Drug	Trospium	Oxybutynin	Tolterodine	Trospium
Dose (x/day)	20 mg × 2	5 mg × 3	0.1 mg/kg	20 mg × 2
Number of patients	52	43	12	29
CC _{max}	increased	increased	increased	increased
Compliance	no change	no change	increased	increased
Pdet _{max} -filling	–	–	decreased	decreased
Pdet _{max}	decreased	decreased	–	–
Q _{max}	–	–	–	no change
Residual volume	increased	increased	–	no change

TABLE 2 Details of the papers reporting results in group II

Variable	[13]	[13]	[13]	[13]	[13]	[13]	[14]	[15]	[16]
Drug	Oxy	Oxy	Oxy	Tol	Tol	Tol	Oxy	Tol	Darifenacin
Dose, mg (x/day)	5 × 3	5 × 3	5 × 3	2 × 2	2 × 2	2 × 2	5 × 2	2 × 2	2.5/10 stat
Comment*	G2	G3	G4	G2	G3	G4			
No. of patients	25	36	40	25	36	40	145	25	18
FDV	inc	inc	inc	NC	inc	NC	inc	–	–
VFOC	inc	inc	NC	NC	inc	inc	–	inc	inc
PFOC	NC	NC	NC	NC	NC	NC	–	–	NC
Pdet _{max} -OC	–	–	–	–	–	–	–	dec	NC
CC _{max}	NC	NC	inc	NC	NC	NC	inc	inc	–
Pdet _{max}	–	–	–	–	–	–	–	NC	–
Q _{max}	–	–	–	–	–	–	–	NC	–
Residual volume	–	–	–	–	–	–	NC	NC	–

*G refers to a subclassification of patients with OAB determined by their volume and pressure of first OC, i.e. G2, high-volume (>250 mL)/high-pressure (>25 cmH₂O); G3, low-volume (<250 mL)/low-pressure (<25 cmH₂O); G4, low-volume (<250 mL)/high-pressure (>25 cmH₂O). NC, no change; inc, increased; dec, decreased; Oxy, oxybutynin; Tol, Tolterodine.

subdivision in data would occur. In view of the limitations in this approach, the data from each article were deemed to be either significant or not, based upon $P < 0.05$, and descriptive analysis used to compare results between articles.

ANALYSIS OF PUBLISHED DATA

For the purpose of this analysis the published articles were divided into three groups depending on the patient group described, i.e. 'neuropaths', 'idiopaths' and mixed (or not specifically stated), labelled groups I to III, respectively.

In group I (Table 1) data were reviewed from three articles [10–12], one of which contained

two treatment groups [10], in all making four reported treatment arms. During bladder filling all treatment arms commented upon an increase in maximum cystometric capacity (CC_{max}), with two arms both showing a significant decrease in maximum Pdet (Pdet_{max}-filling). The effects on compliance were equivocal, with two arms showing no significant change and two a significant increase. In reported voiding variables, Pdet_{max} was significantly decreased in two arms (both from the same article). There was no significant change in maximum urinary flow (Q_{max}) and the effects on residual volume were equivocal.

In group II (Table 2) data were reviewed from three articles and one abstract [13–16]. One

TABLE 3 Details of the papers reporting results in group III

Variable	Jonas <i>et al.</i> [17]	Cardoza <i>et al.</i> [18]	Thüroff <i>et al.</i> [19]	Wagg <i>et al.</i> [20]	Goode <i>et al.</i> [21]	Griffiths <i>et al.</i> [22]	Abrams <i>et al.</i> [23]
Drug	Tolterodine	Trospium	Oxybutynin	Tolterodine	Oxybutynin	Oxybutynin	Tolterodine
Dose, mg (x/day)	2 × 2	20 × 2	5 × 3	2 × 2	IT	2.5–5 × 2	2 × 2
No. of patients	99	132	63	61	105	59	149
FDV	inc	–	–	–	NC	–	–
SDV	–	–	–	–	inc	–	–
VFOC	inc	inc	inc	–	–	–	inc
PFOC	–	NC	–	–	–	–	–
Pdet _{max} -OC	NC	–	–	–	–	–	–
CC _{max}	inc	inc	inc	inc	inc	–	inc
Compliance	inc	NC	–	–	–	–	–
PdetQ _{max}	–	–	–	NC	–	NC	NC
Q _{max}	NC	–	–	–	–	NC	NC
Residual volume	inc	inc	inc	inc	–	inc	inc
Pdet-UO	–	–	–	NC	–	–	–
Pdet-UC	–	–	–	NC	–	–	–

IT, individually titrated; NC, no change; inc, increased.

article, the only article to do so, subdivided patients with OAB into four categories depending on the volume and magnitude of the first overactive contraction (OC) [13]. Patients were labelled as high-volume/low-pressure, high-volume/high-pressure, low-volume/low-pressure or low-volume/high-pressure, depending on the volume and pressure of the first OC in relation to the thresholds 250 mL and 25 cmH₂O, respectively. This subdivision was used to assess two antimuscarinics, resulting in eight treatment arms. However, as two of these arms had too few patients, only six from this article were available for analysis, with one each from the remaining three.

In group II the effects of antimuscarinics on variables associated with the storage phase were not clear. First desire to void (FDV) was significantly increased in five treatment arms, with two reporting no significant change. This is mirrored by the volume at first OC (VFOC), with six showing a significant increase and two no change. Interestingly, in group II, CC_{max} was only significantly increased in three of the arms, and not significantly changed in five, conflicting with data from group I. The Pdet_{max} of the largest OC (Pdet_{max}-OC) again was equivocal, with one arm showing a significant decrease and one no significant change. However, in all groups reporting the pressure of the first OC (PFOC), there was no significant change.

The effects on variables associated with the voiding phase were unequivocal, although relatively few arms were available for analysis. There were no significant changes in either Pdet_{max}, Q_{max} or residual volume.

In group III (Table 3) data were reviewed from six articles [17–22] and one abstract [23]. Group III contained a combination of mixed patient groups and groups where the aetiology was not commented upon. Each article contained one treatment arm examining the effect of an antimuscarinic in current routine use. (One treatment arm containing data for an older medication, propantheline, was not analysed). In one article [21], antimuscarinic and behavioural modification therapy was compared solely with behavioural modification. Although the results for the effects of antimuscarinics were not directly presented, they can be deduced from the results.

In this mixed group, antimuscarinics significantly increased both the VFOC and CC_{max}, suggested by four and six articles, respectively. In addition, the PFOC and the Pdet_{max}-OC were not significantly changed, although only one article each described these findings.

There were no conflicting results in the voiding variables. Three articles reported no significant change in Q_{max} or PdetQ_{max}; six

suggested an increase in residual volume, and in one there was no significant change in Pdet at either urethral opening (UO) or closure (UC).

In general there was a uniform trend between groups II and III, but the main exception to an overall trend was the effect on reported Pdet in group I (neuropaths), with two arms from the same article showing a significant decrease in Pdet during both filling and emptying. This might be a result of the few patients involved or signify differences in the aetiologies of 'idiopathic' and 'neuropathic' OAB.

In examining the data as a whole, a more convincing view emerges as to the effects of antimuscarinics on urodynamic findings. Variables associated with storage, e.g. FDV and CC_{max}, are significantly greater in the vast majority of articles, with variables associated with voiding, e.g. PdetQ_{max} and Q_{max} not being significantly changed. However, the effect on Pdet_{max} seems equivocal, with two articles suggesting a decrease and one suggesting no significant change.

Descriptive analysis of the data allows some conclusions to be formulated for most urodynamic variables, as either the results are consistent among articles, or in the case of some disparity, the vast majority suggest one variable over another. In cases of equivocal

results, articles were divided between either similar changes or no change. There were no cases of opposing results between articles, i.e. a decrease and an increase.

DISCUSSION

For years antimuscarinics have been an integral part of the treatment of DO/OAB. Being designed to reduce the strength of OCs, they were confirmed to do so in higher doses and in animal models. However, in the clinical setting they were found to be therapeutically effective at lower doses. With this success in treatment and with a simple model of how this might be achieved, there was little reason to question the true effects on the bladder. However, with recent increasing anecdotal evidence to suggest an additional effect on sensory symptoms [24], their exact actions have now started to come under scrutiny. Data obtained through this analysis show the sensory effects of antimuscarinics, but more importantly there are no data to support a significant reduction in detrusor contraction in the idiopathic group of patients. There was some reduction in the neuropathic group, but this was limited to results in one article. This anomaly might be a result of the few articles involved overall, or the different causes of OAB, especially as most neuropaths generally have a higher Pdet, which might be more sensitive to the effects of antimuscarinics.

These findings do not dispute the effects of antimuscarinics at higher doses, but suggest that at clinically relevant doses alternative effects need to be considered. The effects upon sensation are strongly supported, but given the lack of data available, more work is required into the effects of therapeutic doses of antimuscarinics upon contraction. Also, a further question remains; 'how do antimuscarinics, and therefore muscarinic receptors within the bladder, alter sensation?'

CONCLUSION

The data currently available do not support the view that antimuscarinics have a significant effect upon bladder contractility in patients with idiopathic DO, but suggest that the benefits seen in these patients are due to improvements in sensory variables.

ACKNOWLEDGEMENTS

We acknowledge the support of Novartis in facilitating discussions which led to the preparation of this manuscript.

CONFLICT OF INTEREST

None declared.

REFERENCES

- Abrams P, Cardozo L, Fall M *et al*. The standardisation of terminology of lower urinary tract function: report from the Standardisation Sub-Committee of the International Continence Society. *Neurourol Urodyn* 2002; **21**: 167–78
- Milsom I, Abrams P, Cardozo L, Roberts RG, Thuroff J, Wein AJ. How widespread are the symptoms of an overactive bladder and how are they managed? A population-based prevalence study. *BJU Int* 2001; **87**: 760–6
- Stewart WF, Herzog A, Wein A *et al*. Prevalence and impact of overactive bladder in the US. Results from the NOBLE program. *Neurourol Urodyn* 2001; **20**: 406
- Hashim H, Abrams P. Do symptoms of overactive bladder predict urodynamics detrusor overactivity? *Neurourol Urodyn* 2004; **23**: 484–6
- Andersson KE, Wein AJ. Pharmacology of the lower urinary tract: basis for current and future treatments of urinary incontinence. *Pharmacol Rev* 2004; **56**: 581–631
- Mansfield KJ, Liu L, Mitchelson FJ, Moore KH, Millard RJ, Burcher E. Muscarinic receptor subtypes in human bladder detrusor and mucosa, studied by radioligand binding and quantitative competitive RT-PCR: changes in ageing. *Br J Pharmacol* 2005; **144**: 1089–99
- Yoshida M, Miyamae K, Iwashita H, Otani M, Inadome A. Management of detrusor dysfunction in the elderly: changes in acetylcholine and adenosine triphosphate release during aging. *Urology* 2004; **63** (Suppl. 3): 17–23
- Kim Y, Yoshimura N, Masuda H, de Miguel F, Chancellor MB. Antimuscarinic agents exhibit local inhibitory effects on muscarinic receptors in bladder afferent pathways. *Urology* 2005; **65**: 238–42
- Yokoyama O, Yusup A, Miwa Y, Oyama N, Aoki Y, Akino H. Effects of tolterodine on an overactive bladder depends on suppression of C-fiber bladder afferent activity in rats. *J Urol* 2005; **174**: 2032–6
- Madersbacher H, Stöhrer M, Richter R, Burgdörfer H, Hachen HJ, Mürtz G. Trospium chloride versus oxybutynin: a randomized, double-blind, multicentre trial in the treatment of detrusor hyperreflexia. *Br J Urol* 1995; **75**: 452–6
- Goessl C, Sauter T, Michael T, Bergé B, Staehler M, Miller K. Efficacy and tolerability of tolterodine in children with detrusor hyperreflexia. *Urology* 2000; **55**: 414–8
- Stöhrer M, Bauer P, Giannetti BM, Richter R, Burgdörfer H, Mürtz G. Effect of trospium chloride on urodynamic parameters in patients with detrusor hyperreflexia due to spinal cord injuries. A multicentre placebo-controlled double-blind trial. *Urol Int* 1991; **47**: 138–43
- Giannitsas K, Perimenis P, Athanasopoulos A, Gryftopoulos K, Nikiforidis G, Barbalias G. Comparison of the efficacy of tolterodine and oxybutynin in different urodynamic severity grades of idiopathic detrusor overactivity. *Eur Urol* 2004; **46**: 776–83
- Madersbacher H, Halaska M, Voigt R, Alloussi S, Höfner K. A placebo-controlled, multicentre study comparing the tolerability and efficacy of propiverine and oxybutynin in patients with urgency and urge incontinence. *BJU Int* 1999; **84**: 646–51
- Athanasopoulos A, Gyftopoulos K, Giannitsas K, Fisis J, Perimenis P, Barbalias G. Combination treatment with an alpha-blocker plus an anticholinergic for bladder outlet obstruction: a prospective randomised, controlled study. *J Urol* 2003; **169**: 2253–6
- Rosario DJ, Cutinha PE, Chapple CR, Milroy E. The effects of single dose darifenacin on cystometric parameters and salivary flow in patients with urge incontinence secondary to detrusor instability. *Eur Urol* 1996; **30** (Suppl. 2): 250, A896
- Jonas U, Höfner K, Madersbacher H, Holmdahl TH. Efficacy and safety of two doses of tolterodine versus placebo in patients with detrusor overactivity and symptoms of frequency, urge incontinence, and urgency: urodynamic evaluation. The International Study Group. *World J Urol* 1997; **15**: 144–51

- 18 Cardozo L, Chapple CR, Toozs-Hobson P *et al.* Efficacy of trosipium chloride in patients with detrusor instability: a placebo-controlled, randomised, double blind, multicentre clinical trial. *BJU Int* 2000; **85**: 659–64
- 19 Thüroff JW, Bunke B, Ebner A *et al.* Randomised, double-blind, multicentre trial on treatment of frequency, urgency and incontinence related to detrusor hyperactivity: oxybutynin versus propantheline versus placebo. *J Urol* 1991; **145**: 813–7
- 20 Wagg A, Malone-Lee J. Pressure-flow variables in patients treated with tolterodine for detrusor overactivity. *BJU Int* 2003; **92**: 969–71
- 21 Goode P, Burgio KL, Locher JL, Umlauf MG, Llyod KL, Roth DL. Urodynamic changes associated with behavioural and drug treatment of urge incontinence in older women. *J Am Geriatr Soc* 2002; **50**: 808–16
- 22 Griffiths D, Harrison G, Moore K, McCracken P. Long-term changes in urodynamic studies of voiding in the elderly. *Urol Res* 1994; **22**: 235–8
- 23 Abrams P, Kaplan S, Millard R. Safety of tolterodine in men with outlet obstruction and symptomatic detrusor overactivity. *Eur Urol* 2002; **41**: Abstract 520
- 24 Andersson KE, Yoshida M. Antimuscarinics and the overactive detrusor – which is the main mechanism of action? *Eur Urol* 2003; **43**: 1–5

Correspondence: James I. Gillespie, The Urophysiology Research Group, School of Surgical and Reproductive Sciences, The Medical School, The University, Newcastle upon Tyne, UK.
e-mail: j.i.gillespie@ncl.ac.uk

Abbreviations: FDV, first desire to void; SDV, strong desire to void; CC_{max}, maximum cystometric capacity; OC, overactive contraction; V(P)F(OC), volume (pressure) at which first (OC) occurs; Pdet_{max} (filling) (OC) maximum detrusor pressure during voiding (during filling phase) [of largest OC]; Q_{max}, maximum flow during voiding; Pdet (Q_{max}) (UO) (UC), detrusor pressure at Q_{max} (at urethral opening) (urethral closure).

Cholinergic activation of phasic activity in the isolated bladder: possible evidence for M_3 - and M_2 -dependent components of a motor/sensory system

Steven M. Finney, Laurence H. Stewart and James I. Gillespie*

Western General Hospital, Edinburgh, Scotland and *The Urophysiology Research Group, School of Surgical and Reproductive Sciences, The Medical School, The University, Newcastle upon Tyne, UK

Accepted for publication 2 March 2007

OBJECTIVES

To analyse pressure changes induced by muscarinic agonists on the isolated bladder in order to examine whether there are different responses representing different components of a motor/sensory system within the bladder wall.

MATERIALS AND METHODS

Whole isolated bladders from 19 female guinea-pigs (280–400 g) were used. A cannula was inserted into the urethra to monitor intravesical pressure and the bladder was suspended in a heated chamber containing carboxygenated physiological solution at 33–36 °C. Initially, the responses to the cholinergic agonists, arecaidine but-2-ynyl ester tosylate and carbachol were assessed. Then, in an attempt to identify the muscarinic receptor subtypes involved, the effects of selective muscarinic antagonists on the arecaidine-induced bladder responses were assessed. The antagonists used were the relatively M_3 -selective 4-diphenylacetoxy-N-methylpiperidine methobromide (4-DAMP) and darifenacin, and relatively M_2 -selective AFDX-116. All drugs were added to the solution bathing the abluminal surface of the bladder.

RESULTS

The whole bladders exposed to cholinergic agonists respond with complex changes in intravesical pressure. Immediately after application of the agonist there was a burst of high frequency transient contractions. During continued application of agonist the frequency of the transients decreased and their amplitude increased. Thus, there appear to be two components to the response: an initial fast phase and a later slow component. The maximum frequency of the initial burst increased with increasing concentrations of agonist. By contrast, the frequency of the transients in the steady state showed little dependence on agonist concentration. There were quantitative differences between the responses to arecaidine and carbachol. Arecaidine was less effective in generating the initial burst of high-frequency activity and the transients were significantly larger. At low dose, arecaidine was more effective in producing the large transients in the steady state. Pre-exposure of the bladder to 4-DAMP (0.1–10 nM) or darifenacin (0.1–10 nM) significantly reduced the frequency of the initial burst of activity; 0.3 nM 4-DAMP reduced the frequency by half. In this concentration range, 4-DAMP reduced the amplitude of the initial transients but did not affect the frequency of the transients in the steady state. There were similar results with darifenacin. However, darifenacin was less

effective in reducing the amplitude of the initial transients. By contrast, AFDX-116 had little effect on the frequency of the initial transients but did reduce amplitude; 300 nM AFDX-116 was needed to reduce the frequency of the initial burst by half.

CONCLUSIONS

This analysis suggests that there are different but interrelated mechanisms in the isolated bladder contributing to complex contractile activity. Three components can be identified: a mechanism operating during voiding to produce a global contraction of the whole bladder and two mechanisms, pacemaker and conductive, involved in generating and propagating local contractions in the bladder wall. The pacemaker component is more sensitive to darifenacin and 4-DAMP than to AFDX-116 suggesting that the underlying processes rely predominantly on M_3 receptors and less so on M_2 ($M_3 > M_2$). The phasic activity in the later stages is less affected by M_3 antagonists and might therefore involve predominantly M_2 receptors ($M_2 > M_3$). The potential importance of these results in terms of the general physiology and pharmacology of the bladder is discussed.

KEYWORDS

phasic activity, 4-DAMP, AFDX-116, darifenacin

INTRODUCTION

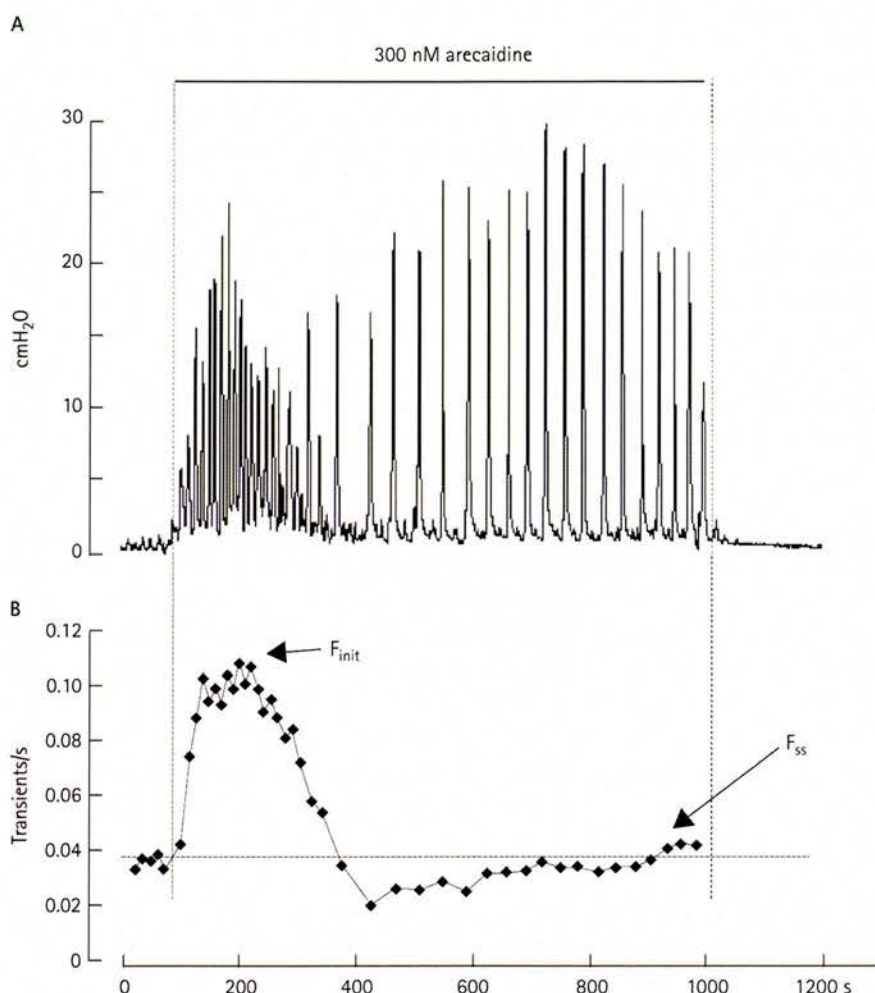
Incontinence, increased urge and increased frequency of micturition affect nearly 100 million people in the western world [1,2]. These LUTS are not life-threatening but they seriously affect quality of life and ability to work. The primary clinical problem is an

increased urge to pass urine [3]. Remarkably, despite the prevalence of LUTS the mechanisms underlying increased urge are not known. To simply describe the condition the term 'Over Active Bladder (OAB)' symptom complex has been introduced [1]. In many cases, studied using conventional urodynamics, sensations of urge are correlated with rises in intravesical

pressure, a condition classified as detrusor overactivity (DO). In the remainder of patients, sensation is not accompanied by abnormal contractions, a condition previously described as sensory urgency [1–4]

As the DO component of OAB is associated with contractions in the bladder, it was

FIG. 1. An example of the effect of adding 300 nM arecaidine to an isolated guinea-pig bladder. **A**, the original record of intravesicle pressure, the horizontal bar represents arecaidine addition to the bathing solution. **B**, shows an analysis of this record. The instantaneous frequency of the transients (reciprocal of the time interval between each transient) is plotted against the time of the second of each pair. This shows how the agonist affects the frequency with time of application. Two measurements used in subsequent analysis are indicated by the arrow. Experiment was done at 34 °C



argued that drugs affecting contractility would alleviate symptoms. Activity in the bladder smooth muscle is initiated at muscarinic receptors (M_3). Drugs, designed to target these M_3 receptors on the muscle, have proven effective in decreasing urge and incontinence. However, it is emerging that, at therapeutic doses, these drugs do not significantly affect bladder contraction [5–7]. In addition, anticholinergic drugs are effective in reducing symptoms of urge in those patients who do not have DO as determined by medium-filling rate cystometry [5–7]. If the anticholinergics are only affecting the uncontrolled contractions of the detrusor these patients should not benefit from

anticholinergic drug therapy. Thus, we are coming to the inescapable conclusion that the site of action of these anticholinergic drugs is unknown.

The anticholinergic drugs must work on cholinergic mechanisms within the bladder or lower urinary tract that are not immediately associated with the generation of the voiding contraction or the activity described as overactive contractions. As they influence sensation, it is possible that they affect cholinergic mechanisms associated with the generation of afferent signals in the bladder [5–10]. The nature of these additional mechanisms and the cells expressing the

muscarinic receptors are not known. Therefore, the search is on to identify these novel cholinergic mechanisms in the bladder and ascertain if they are the therapeutic targets for the anticholinergic drugs.

Studies on the isolated whole bladder preparation suggested that there might be distinct cholinergic mechanisms that can result in different forms of detrusor activity [11,12]: localized phasic activity consisting of propagating waves of contraction with local stretches and global synchronous contractions. It has been proposed that the phasic activity plays a role in the generation of afferent signals from the bladder [13,14]. Potentially, this mechanism could be a candidate for the therapeutic action of the anticholinergic drugs. Therefore, it is crucial to examine in detail the cholinergic mechanisms resulting in the activation of phasic activity.

MATERIALS AND METHODS

The procedures for isolating the whole bladder and maintaining it *in vitro* have been described [11,12]. Briefly, 19 female guinea-pigs (280–400 g) were killed by cervical dislocation in accordance with schedule 1 of the UK Home Office regulations. The urinary bladder and urethra were removed and placed in Tyrodes solution (composition in mM: NaCl, 120; KCl 4.5; CaCl₂ 2.5; MgCl₂ 1; NaHCO₃ 25; NaH₂PO₄ 1; Na pyruvate 1, glucose 5) bubbled with 5% CO₂ and 95% O₂ (pH 7.4). The mean (SD) bladder weight was 303 (55) mg (19 bladders). A flexible plastic cannula (2 mm diameter) was inserted into the urethra and secured at the bladder neck using a thread ligature. Residual urine was removed and replaced with Tyrodes solution. The bladder was transferred to a heated organ bath (30 mL at 33–36 °C) containing constantly gassed Tyrodes solution. The cannula was connected with the urethra uppermost via a fluid-filled tube with a three-way connector to a pressure transducer (model DTX Plus, Becton Dickinson, Oxford, UK) and a 1-mL syringe to enable variation of the intravesical volume. The transducer output was amplified, digitized (sampling rate 10 Hz) and recorded using a capture system (Acqknowledge AD, Newcastle Photometrics Systems, Newcastle, UK). The transducer was calibrated before each experiment. At the beginning of each experiment, each bladder was filled with Tyrodes to give a baseline volume of

1000–1200 μL . These volumes were typically the amount of urine recovered from the guinea-pigs immediately after cervical dislocation. The time taken from killing to the beginning of recording was typically 30 min. Pressure recording was begun immediately but the bladder was left to equilibrate for at least 20 min before any manipulations.

Initially, the responses of the isolated bladder to cholinergic agonists, arecaidine but-2-ynyl ester tosylate (Tocris, Avonmouth, UK) and carbachol (Sigma, St. Louis, MO, USA), were assessed. Then, in an attempt to identify the muscarinic receptor subtypes involved, the effects of selective muscarinic antagonists on the arecaidine-induced bladder responses were assessed. The antagonists used were the relatively M_3 -selective 4-diphenylacetoxy-N-methylpiperidine methobromide (4-DAMP: $M_3 > M_2$, 9 : 1) and darifenicin ($M_3 > M_2$, 60 : 1) [15], and relatively M_2 -selective AFDX-116 ($M_2 > M_3$, 4 : 1). The concentrated drug solutions were added directly to the solution bathing the serosal/abluminal surface of the isolated whole bladder to achieve the required final dilution.

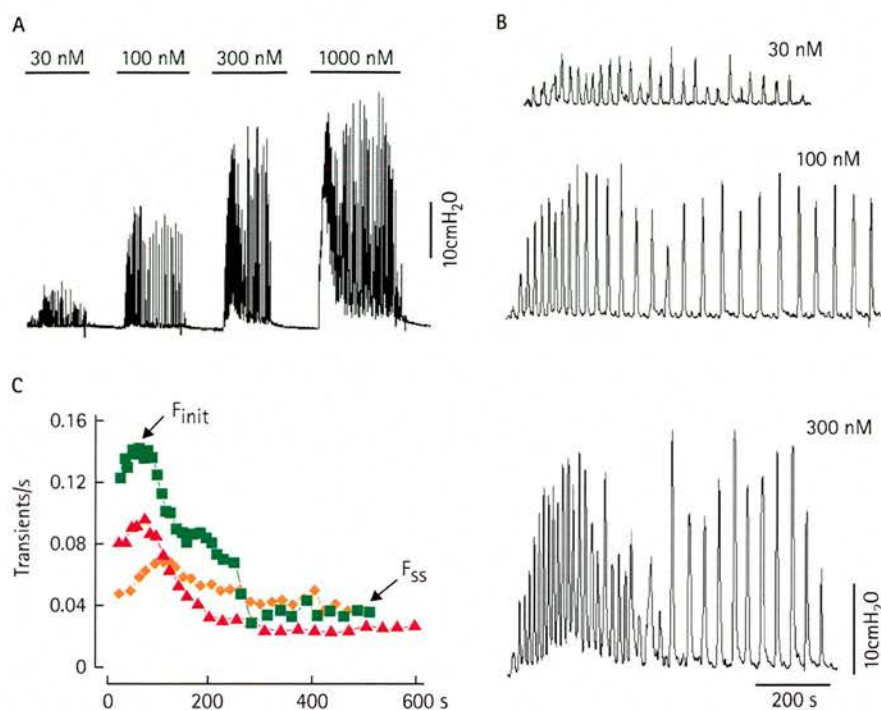
The quantitative values are given as the mean (SD). The differences between means were determined by a paired *t*-test and the level for significance set at 0.05. The number of preparations used in the statistical analysis for each experiment and each condition are given in the appropriate figure legends.

RESULTS

RESPONSES OF THE ISOLATED BLADDER TO ARECAIDINE AND CARBACHOL

Whole bladders, exposed to cholinergic agonists, respond with complex changes in intravesical pressure. Figure 1A shows an example of a response induced by the application of 300 nM arecaidine; immediately upon application a series of transient rises in intravesical pressure occur. Figure 1B shows an analysis of this activity. The instantaneous frequency of the transients (calculated as the reciprocal of the interspike interval) is plotted against time of the exposure. In the early stages of the response the transients increase in amplitude and frequency to a maximum. The maximum frequency reached in this initial phase is assigned the term F_{init} . The frequency of the transients and their amplitude then decline. After this phase both the amplitude and

FIG. 2. The effects of different concentrations of arecaidine on the induced transients in an isolated whole bladder. **A**, a section of the original record. **B**, three sections of the record on an expanded time scale: 300 nM (square), 100 nM (triangle) and 30 nM (diamond). **C**, an analysis of the records in B showing the instantaneous frequency of the first 600 s of the records. The two secondary measurements are shown, F_{init} and F_{ss} . Experiment done at 34 °C.



frequency of the transients increase eventually reaching a steady value F_{ss} . The amplitude of these spikes reaches a relatively constant magnitude. In subsequent analyses the magnitudes of F_{init} , F_{ss} , and the maximum pressure change of the transients in the initial and steady-state phases are calculated.

Figure 2 shows the effects of different concentrations of arecaidine; as the concentration was increased F_{init} increased but there was little difference in F_{ss} . The maximum value of the amplitude of the transients in the initial phase and in the steady state both increased with agonist concentration. Qualitatively similar responses were obtained with carbachol (Fig. 3). However, there were specific differences in these responses. At similar concentrations, the magnitude of F_{init} was greater with carbachol and the transients in the steady state were more variable in amplitude and frequency. The combined data from 14 bladders is shown in Figure 4. The ability of carbachol to generate higher frequencies in the initial phase is clearly seen (Fig. 4A). The lack of any significant effect of different

concentrations of arecaidine or carbachol on the frequency of the transients in the steady state is also apparent (Fig. 4B). Arecaidine was more effective, at similar doses, in increasing the amplitude of the transients in both the initial and steady-state phases (Fig. 4C,D).

M_3 -SELECTIVE ANTAGONISTS

In each experiment, an initial control response to agonist was elicited. During the subsequent wash period, 4-DAMP was added to the bathing solution. After a further 5 min a second exposure to the same concentration of agonist was given and the effect of 4-DAMP determined. Upon washing off the agonist for this response the concentration of 4-DAMP was increased. This sequence was then repeated for increasing concentrations of 4-DAMP. Figure 5A shows the original records from a bladder treated with 4-DAMP; six bladders were studied using this protocol. Figure 5B shows sections of the record in A on expanded time scales. Close examination of these responses show that F_{init} was reduced with increasing concentrations of 4-DAMP while there was little effect on the amplitude.

FIG. 3. The effects of different concentrations of carbachol on the induced transients in an isolated whole bladder. **A**, a section of the original record. **B**, three sections of the record on an expanded time scale: 1000 nM (triangle), 300 nM (circle) and 100 nM (square). **C**, an analysis of the records in B showing the instantaneous frequency of the first 400 s of the records. The two secondary measurements are shown, F_{init} and F_{ss} . Experiment done at 34 °C.

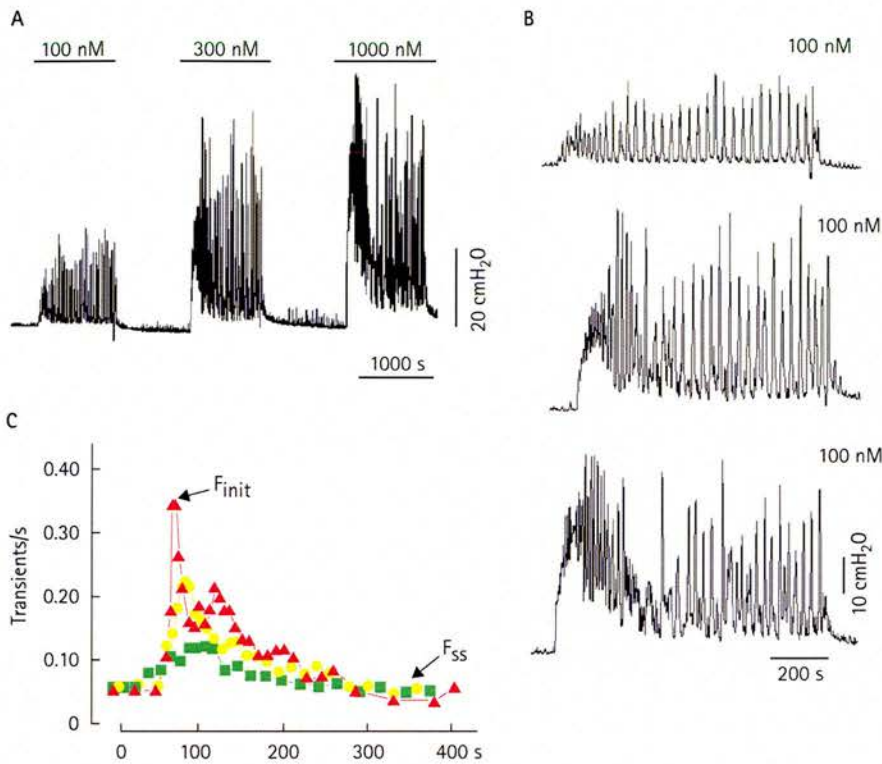
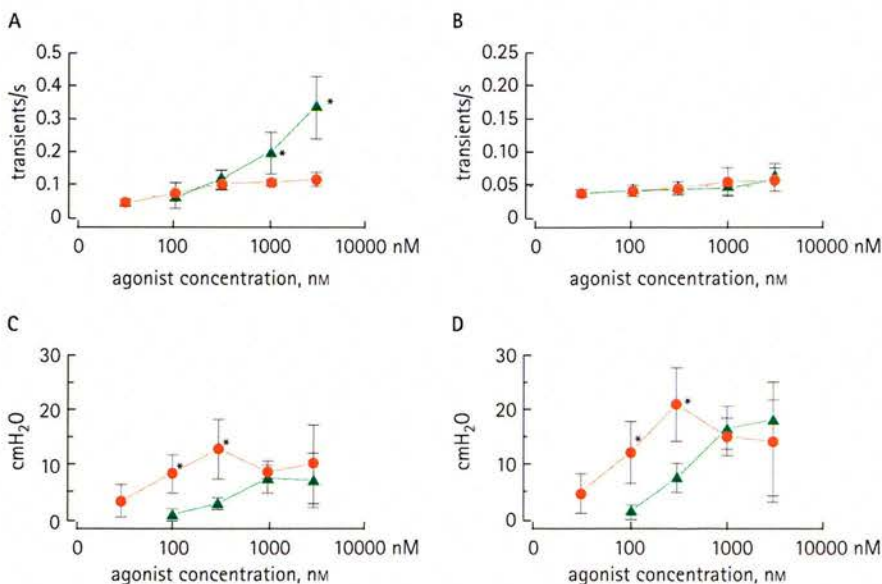


FIG. 4. An analysis of responses from different bladders to arecaidine (circle; eight bladders) and carbachol (triangle; six bladders). **A** and **B** show, respectively, F_{init} and F_{ss} during exposure to different concentrations of arecaidine or carbachol. **C** and **D** show, respectively, the variations in the amplitude of the transients during the initial phase and late phase of the responses. * $P < 0.05$ carbachol vs arecaidine (student t-test). Temperature of experiments 33–34 °C.



Also, there was little effect on F_{ss} . The changes in instantaneous frequency for each 4-DAMP concentration is shown in Figure 5C; as the concentration of 4-DAMP increased the F_{init} decreased while the F_{ss} was little changed.

Figure 6 shows data collected from six experiments. The effects of 4-DAMP on F_{init} and F_{ss} are shown in A and B, respectively. At low dose (0.3 nM) there was a significant reduction of F_{init} . As the concentration of 4-DAMP increased the reduction in F_{init} decreased further, appearing to reach a minimum value at ≈ 10 nM 4-DAMP of 0.07 transients/s. By contrast, the effect of 4-DAMP on the F_{ss} was less pronounced. Interestingly, the lowest value of F_{init} at 10 nM 4-DAMP (0.07 transient/s) was approaching the values of F_{ss} . Thus, it is possible that there is a component of the transients in the initial response that reflects the mechanism in the steady state. If this is so then the initial component sensitive to 4-DAMP is almost completely inhibited by 3 nM 4-DAMP. Concentrations of 4-DAMP as low as 0.3 nM being effective in reducing this component by half.

Darifenicin was also effective in reducing the phasic arecaidine-induced activity (Figs 7 and 8). As with 4-DAMP darifenicin reduced F_{init} but had little effect of F_{ss} . The amplitude of the transients in the initial phase and in the steady state were also reduced by darifenicin. The reduction in F_{init} with 4-DAMP and darifenicin were very similar. However, there appeared to be a difference in the effect of 4-DAMP and darifenicin on the amplitude of the initial transients. This difference is shown in Figure 9 where the data from Fig. 6A and C and Fig. 8A and C are combined. Panel A shows the effects of the two antagonists on the amplitude of the transients. 4-DAMP was more effective at these low concentrations in reducing the amplitude of the initial transients than darifenicin. By contrast, both agonists were equally effective in reducing F_{init} . This could suggest that the mechanisms involved in determining the F_{init} involves M_3 muscarinic receptors. The mechanisms involved in regulating the amplitude of the initial transients were affected more by 4-DAMP than darifenicin. As 4-DAMP has a partial effect on M_2 receptors, which is greater than that of darifenicin the reduction in amplitude could also reflect an involvement of M_2 receptors. That neither 4-DAMP nor darifenicin affected F_{ss} significantly, although also induced by muscarinic exposure,

suggests less involvement of M_3 receptors in F_{ss} .

M_2 -SELECTIVE ANTAGONIST

An example of an experiment showing the effects of AFDX-116 is shown in Figure 10 and the collected data from six bladders shown in Figure 11. Figure 10A shows the entire experiment while panel B shows sections of the record on an expanded time scale. As the concentration of AFDX-116 was increased the amplitude of the transients was reduced. An analysis of the instantaneous frequency changes are shown in Fig. 10C but there appears was little effect of AFDX-116 on F_{init} . The combined data confirms that there is little effect of AFDX-116 on the F_{init} or F_{ss} . Only at the highest concentration (1000 nM) did AFDX-116 significantly reduce F_{init} or F_{ss} . The amplitudes of the initial and steady-state transients were reduced by AFDX-116 at the highest concentrations used (1000 nM).

DISCUSSION

It is widely accepted that the M_3 -selective anticholinergic drugs are effective in reducing the symptoms of OAB. Contraction of the smooth muscle cells in the bladder involves activation of M_3 muscarinic receptors [16]. However, it is unlikely that this population of M_3 receptors are involved in the therapeutic action of anticholinergic drugs [6–10]. It is emerging that the anticholinergic drugs diminish the sensations of urge and so reduce the need for frequent voiding. Thus, the therapeutic site of action of the muscarinic drugs must be on M_3 receptors operating within a system involved in the generation or modulation of sensations during the filling phase of the micturition cycle. Where these receptors are located and what their role is in sensory mechanisms are not known. The search is therefore on to find these M_3 -dependent mechanisms. This paper suggests one possible mechanism.

A further complication concerns the location and function of M_2 receptors in the bladder wall. There are data reporting that there are more M_2 receptors in the bladder than M_3 . Despite this, we do not know what physiological role these receptors serve or where they are located [17–22]. Possible mechanisms involving the M_2 -dependent regulation of detrusor contraction have been suggested. M_2 activation inhibits adenyly

FIG. 5. The effects of the M_3 -selective antagonist 4-DAMP on the responses of an isolated whole bladder preparation in response to repeated exposures to 1000 nM arecaidine. **A**, the whole experiment; the response in the absence of 4-DAMP is described as the control. The responses in the presence of increasing concentrations of 4-DAMP are shown. **B**, shows these different responses on an expanded time scale. **C**, an analysis of the responses showing the changes in the instantaneous frequency of the transients with time for the different concentrations of 4-DAMP: control (square), 1 nM (diamond), 3 nM (triangle) and 10 nM (circle). Temperature of experiments 33–34 °C.

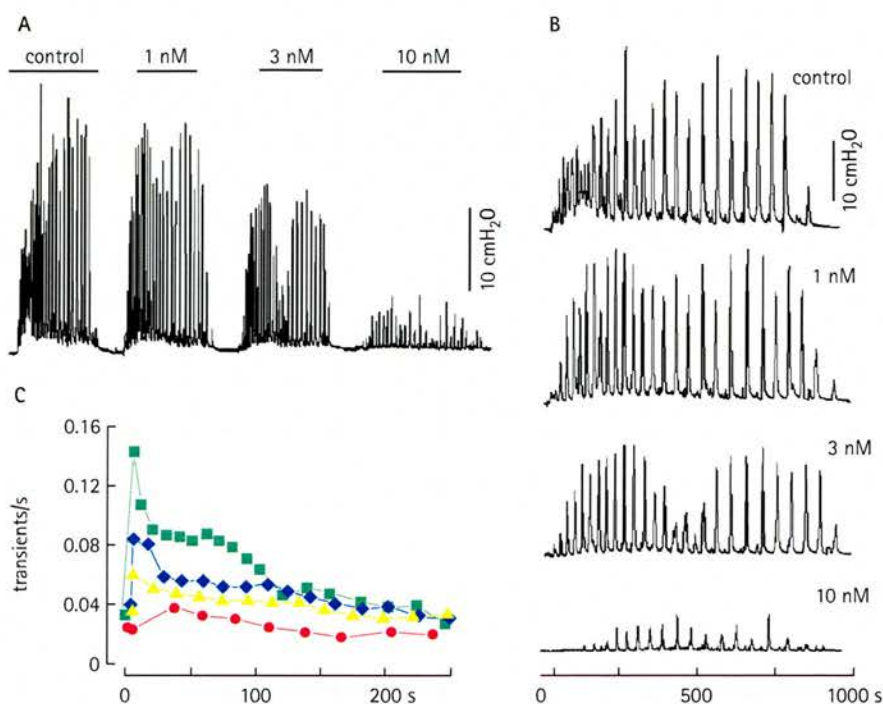


FIG. 6. Data from six bladders showing the effects of different concentrations of the M_3 -selective antagonist 4-DAMP on the arecaidine-induced responses. **A** and **B** show the effects of 4-DAMP on F_{init} and F_{ss} , respectively. **C** and **D** show the effects on the amplitudes of the initial and steady transients, respectively. Data are shown as the mean (SD) for six bladders. * $P < 0.05$ vs control (student t-test).

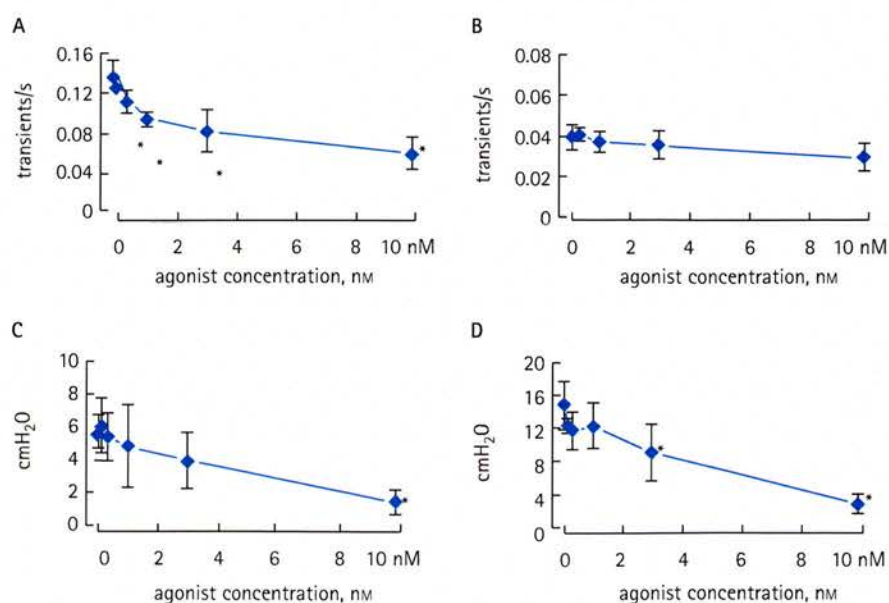


FIG. 7. The effects of the M_3 -selective antagonist darifenacin on the responses of an isolated whole bladder preparation in response to repeated exposures to 1000 nM arecaine. **A**, the whole experiment; the response in the absence of darifenacin is described as the control. The responses in the presence of increasing concentrations of darifenacin are also shown. **B**, shows two of these responses on an expanded time scale. **C**, an analysis of the responses in **B** showing the changes in the instantaneous frequency of the transients with time for the different concentrations of darifenacin: control (red circle) and 0.3 nM darifenacin (blue circle). Temperature of experiments 33–34 °C.

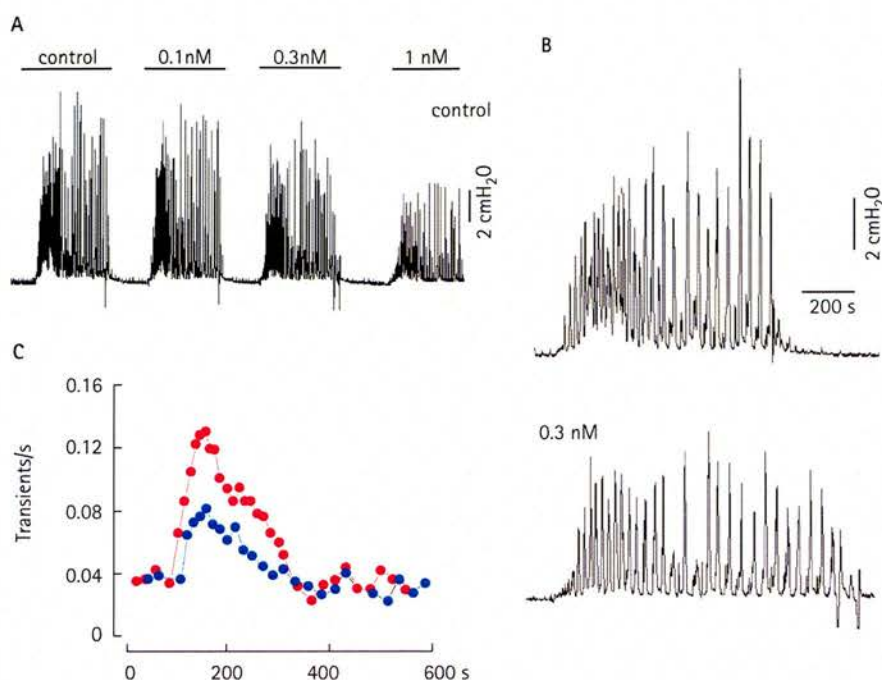
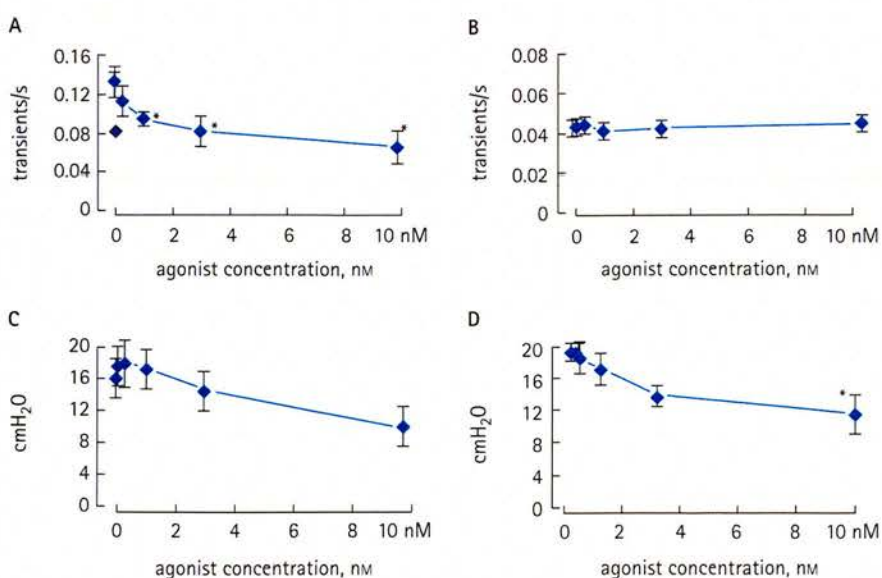


FIG. 8. Data from nine bladders showing the effects of different concentrations of the M_3 -selective antagonist darifenacin on arecaine-induced responses. **A** and **B** show the effects of darifenacin on F_{init} and F_{ss} , respectively. **C** and **D** show the effects on the amplitudes of the initial and steady transients, respectively. Data are shown as the mean (SD) for six bladders. * $P < 0.05$ vs control (student t-test).



cyclase resulting in a decrease in intracellular cAMP. Since a decrease in cAMP leads to an increase in contractility this was proposed as a possible function of M_2 receptors in the regulation of bladder contractility. In addition, as M_2 receptors are more prevalent within the urothelial layer there is a further suggestion that the urothelium might be involved in the regulation of detrusor contractility.

It has been recognized for >120 years that the bladder is not quiescent during the filling phase. Small transient rises in pressure occur that become larger and more frequent as the bladder fills [23–26]. It was proposed that this non-micturition activity generates afferent discharges that contribute to bladder sensation [14,27]. Phasic activity similar to the non-micturition activity seen *in vivo* can be detected in the isolated bladder [14,24]. Using this preparation it was shown that the phasic activity or, as it is known 'autonomous activity', can be augmented by muscarinic agonists and this augmented activity is increased by ATP and substance P [28]; it can also be inhibited by calcitonin gene-related peptide and noradrenaline [29,30]. This has led to the idea that the non-micturition activity can be modulated to alter the sensitivity and output of this sensory/motor system [14]. As the non-micturition activity is influenced by muscarinic stimulation it follows that reduction of this muscarinic component by anticholinergic drugs will reduce sensation. Thus, this mechanism could represent the therapeutic site of action of the antimuscarinic drugs.

The present study shows the responses of the whole isolated bladder to muscarinic stimulation. Exposure to an agonist results in a complex pattern of transient rises in intravesical pressure that change during the period of the application. Immediately the bladder is exposed to agonist the frequency of the transient rises rapidly to a maximum (F_{init}) and then declines to a steady level (F_{ss}). The F_{init} increases with increasing agonist concentration. By contrast, the F_{ss} is not markedly different with different agonist concentrations. At present, the mechanisms that generate and regulate both frequency and amplitude of the phasic activity are not known. It is unlikely that they originate as a result of activation of the M_3 receptors on the detrusor (see [14] for a discussion). However, a detailed analysis and consideration of the changes might give insights into the underlying mechanisms.

It was shown that the transient rises in pressure induced by muscarinic agonists are accompanied by propagating waves of contraction over large parts of the bladder wall and by local stretches [11,12]. This led to the idea that the transients are initiated by a 'pacemaker-like' mechanism and that the activity is then distributed throughout the bladder wall by specialized networks of cells, nerves or interstitial cells (ICs) [13,14]. In the present study, that the F_{init} was dependent on muscarinic agonist stimulation, suggests an involvement of muscarinic receptors on the cells responsible for generating the pacemaker activity.

That the agonist-dependent changes in F_{init} were sensitive to the relatively M_3 -selective muscarinic antagonists 4-DAMP and darifenicin [16], further suggests that the cells performing the pacemaker function express and operate with predominantly the M_3 receptor subtype. The small but clear differences in the actions of 4-DAMP and darifenicin also support this possibility. That the more selective agent, darifenicin, at low concentration, affects the frequency of the transients but not the amplitude suggests that the pacemaker mechanisms is indeed regulated by M_3 receptors while the mechanisms influencing the amplitude are not exclusively so, involving both M_3 and M_2 . The greater decrease in the amplitude of the initial transients with 4-DAMP can be interpreted to suggest that this is the consequence of inhibition of an M_2 component with the less selective antagonist.

The amplitude of the transients was related to the concentration of the muscarinic agonist and the degree of muscle activation resulting from the spread of activity over the bladder surface. This suggests that the propagation of the activity is regulated by a muscarinic mechanism that might be more dominated by M_2 than M_3 receptors. This is supported by the finding that the relatively M_2 -selective antagonist AFDX-116 [31] did not markedly influence the F_{init} . However, AFDX-116 did affect the amplitude of the transients.

The activity generated in the pacemaker mechanism by muscarinic stimulation is transient; the stimulus activates an initial burst of activity that declines to a steady state. As already stated, the F_{init} depends on the applied agonist concentration and is inhibited by 4-DAMP and darifenicin. By

FIG. 9. Summary of the mean data from the series of experiments with 4-DAMP and darifenicin showing the effects of 4-DAMP (blue diamond) and darifenicin (red diamond) on the amplitude (A) of the transients induced by arecaidine and F_{init} (B).

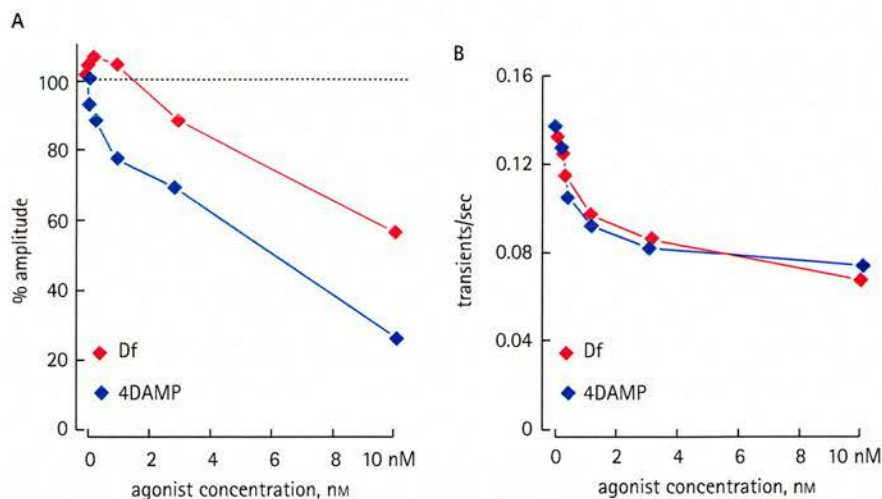


FIG. 10. The effects of the M_2 -selective antagonist AFDX-116 on the responses of an isolated whole bladder preparation in response to repeated exposures to 1000 nM arecaidine. A, a whole experiment; the response in the absence of AFDX-116 is described as the control. The responses in the presence of increasing concentrations of AFDX-116 are also shown. B, shows these different responses on an expanded time scale. C, an analysis of the responses showing the changes in the instantaneous frequency of the transients with time for the different concentrations of AFDX-116: control (triangle), 100 nM (square) and 300 nM (diamond). Temperature of the experiment 33 °C.

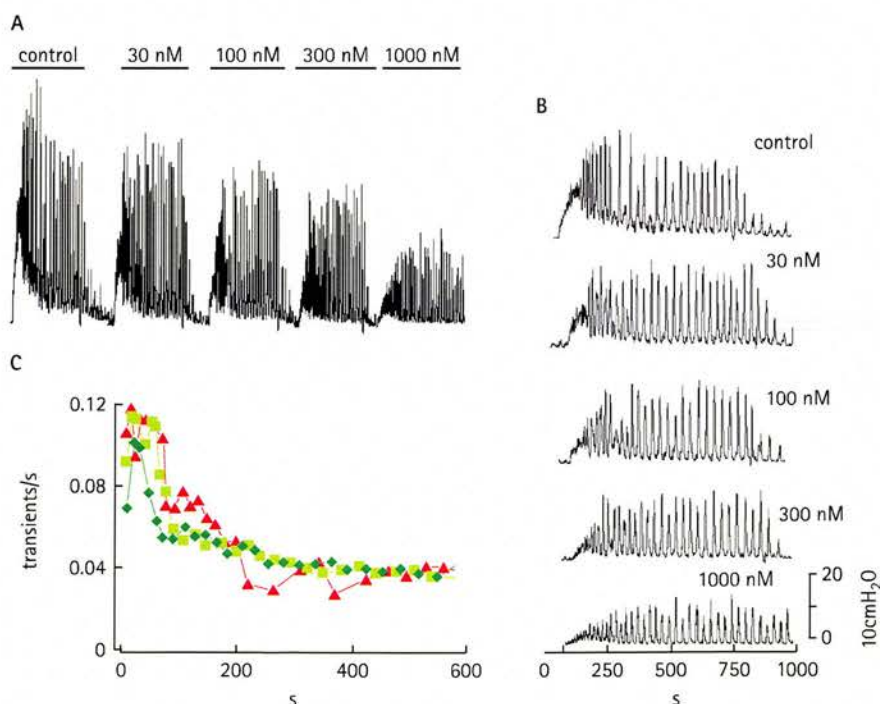
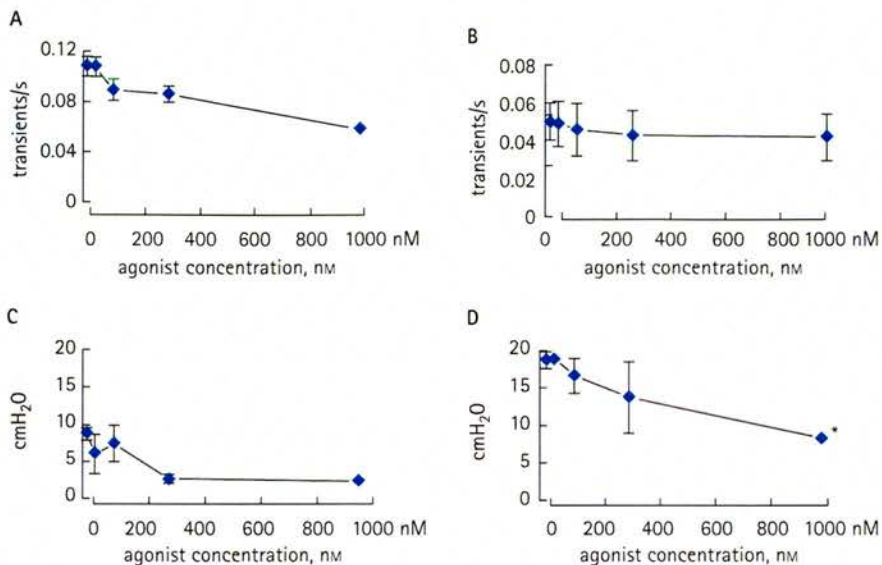


FIG. 11. Data from six bladders showing the effects of different concentrations of the M_2 -selective antagonist AFDX-116 on arecaidine-induced responses. A and B show the effects of AFDX-116 on the F_{int} and F_{ss} , respectively. C and D show the effects on the amplitudes of the initial and steady transients, respectively. Data are shown as the mean (SD) for six bladders. * $P < 0.05$ vs control (student t-test).



contrast, the F_{ss} is less sensitive to agonist concentration and to M_3 inhibition. This might argue that the initial transients and steady-state transients might be dependent upon different but interrelated mechanisms. Indeed, it is possible that there could be two separate mechanisms. In keeping with this idea the nicotinic ligand lobeline was reported to be capable of producing slow large transient rises in pressure similar to those seen in the steady state but did not generate a rise in basal pressure or bursts of high frequency activity [12]. Taken together these data are consistent with the view that there are two mechanisms operating to generate and propagate phasic activity in the guinea-pig bladder: a pacemaker, used to determine the frequency of the phasic activity, and a distribution mechanism used to propagate activity in waves of contraction throughout the bladder. This scheme is illustrated in Figure 12. As the initial burst of activity is activated by muscarinic agonists and inhibited by 4-DAMP and darifenicin, it is likely that this pacemaker mechanism is regulated by predominantly M_3 muscarinic receptors rather than M_2 ($M_3 > M_2$). By contrast, the large steady-state transients are less sensitive to the M_3 antagonists and might be dependent on M_2 receptors operating via changes in intracellular cAMP ($M_2 > M_3$).

That the frequency of the large transients generated during the steady state of an agonist-complex response is relatively insensitive to the M_3 -selective antagonists suggests that cholinergic stimulation of these cells might predominantly be via M_2 receptors. The M_2 -selective antagonist AFDX-116 reduced the amplitude of the transients. The large transients in the steady state were larger and more regular when induced by arecaidine than with carbachol. Arecaidine is a muscarinic agonist with a reported greater selectivity for the M_2 than the M_3 receptor [32]. Thus, ICs might be activated and regulated by M_2 receptors. Activation of M_2 muscarinic receptors in many cells inhibits adenylate cyclase resulting in a decrease in intracellular cAMP [18,19]. If this is correct then activity in the IC is regulated by cAMP: a decrease in cAMP results in an increase in excitability. Consistent with this idea it was shown that procedures that elevate cAMP (forskolin and phosphodiesterase inhibitors) inhibit phasic activity in the isolated bladder preparation [33]. This scheme represents a possible role for M_2 receptors in the bladder.

It must not be forgotten that there must be a further component of bladder activity resulting from direct activation of the M_3 muscarinic receptors on the smooth muscle probably by postganglionic parasympathetic

nerves and involved in the generation of the voiding contraction. Activation of the smooth muscle directly is likely to result in simultaneous activation of all of the muscle resulting in a contraction.

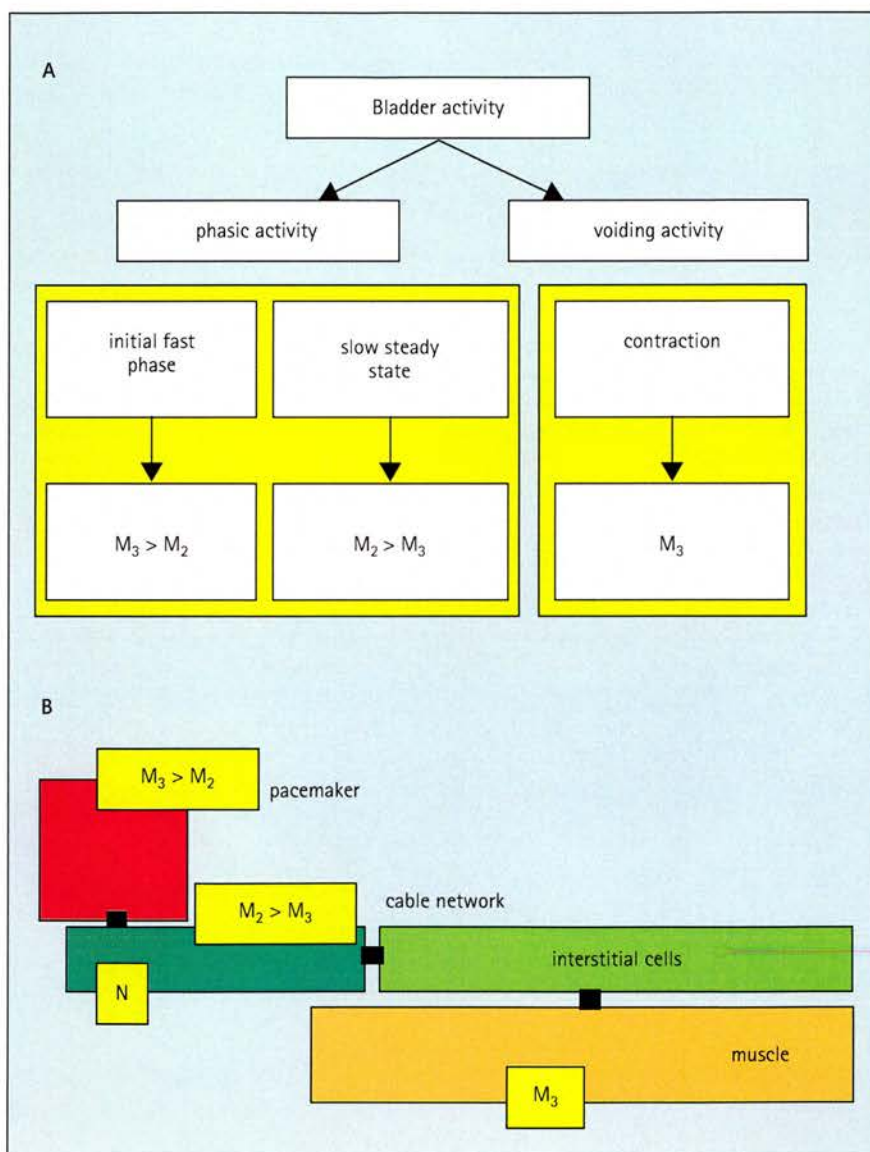
Recent studies identified possible structural correlates of this functional framework. There is growing evidence for the presence of a heterogeneous network of cells, ICs, associated with the detrusor that might subserve the functions of pacemaker and of a coordinating network controlling contraction waves in the detrusor [34–40]. In the guinea-pig and mouse, the responsiveness of ICs to nitric oxide resulting in an elevation in intracellular cGMP has been used to identify subtypes and their distribution [34,37,39,40]. In the guinea-pig three types of ICs have been identified associated with the outer muscle layer: cells running on the outer margin of the bladder wall (muscle coat ICs), cells on the surface of the muscle bundles (surface muscle ICs) and cells within the muscle bundles (intramuscular ICs) [37]. Also, there is some evidence to suggest that the intramuscular ICs might consist of two populations of cells: cells expressing choline acetyl transferase (ChAT) and cells not expressing ChAT [39]. In the mouse there appear to be only two types of IC associated with the outer muscle layers: intramuscular and surface muscle ICs. In both guinea-pig and mouse the surface muscle and intramuscular ICs come into close contact with nerve fibres, suggesting that activity in these cells might be under neural control, possibly cholinergic [39]. In support of this possibility, activity in an isolated bundle of smooth muscle cells originates at specific points on the bundle surface, presumably the ICs [36]. The concept that is evolving is that cholinergic nerve-mediated activity in the network of ICs is transferred to the underlying muscle bundles resulting in coordinated episodic waves of contractile activity. There is a report showing that nitric oxide selectively reduced the phasic activity in the isolated mouse bladder [40]. As the major cell type sensitive to nitric oxide in the mouse bladder are the ICs then this suggests that these cells do play an integral part in the generation of phasic activity.

The location and nature of a potential pacemaker mechanism is not known. In the guinea-pig, a recent study showed the presence of a suburothelial ganglionic network. The neurones in these ganglia are contacted by different types of nerve fibre.

Three types have been identified: fibres expressing calcitonin gene-related peptide, fibres expressing ChAT and neurofilament protein (ChAT/NF⁺) and fibres expressing ChAT but not neurofilament (ChAT/NF⁻) [41]. The calcitonin gene-related peptide and ChAT/NF⁻ fibres might be collaterals of sensory fibres, which appear to originate in the suburothelial layer. The ChAT/NF⁺ fibres might be axons from adjacent ganglia. Furthermore, two types of nerve fibres innervate the muscle layer ChAT/NF⁺ and ChAT/NF⁻ fibres. The ChAT/NF⁺ fibres might also contact ICs in the outer muscle layer. Thus, there is evidence for a suburothelial ganglionic network with the potential to receive inputs from the urothelium and adjacent ganglia and which has outputs to the muscle layers. It has been speculated that such a network might play a role in the local reflexes in the bladder wall linking urothelial distortion to phasic contractile activity [41]. In the context of this discussion it is notable that the suburothelial ganglion cells receive inputs from two types of nerve fibre that are likely to be cholinergic suggesting that the ganglionic cells express cholinergic receptors. Application of exogenous muscarinic agonists might be expected to activate these ganglia. If these ganglia are associated with reflex activation of phasic activity then the direct activation with an exogenous muscarinic agonist will also generate phasic activity. Therefore, there is circumstantial evidence to suggest a role for the suburothelial ganglionic network, the pacemaker, and the different types of ICs, a heterogeneous network carrying pacemaker activity to the muscle layer.

In summary, the present study using the isolated guinea-pig bladder, shows that the rapid component of the phasic activity recorded is generated and its frequency determined by activation of muscarinic M₃ receptors. This pacemaker activity is particularly sensitive to M₃-selective antagonists at subnanomolar concentrations. These concentrations are unlikely to affect the voiding contraction. Thus, we have a functional target for low doses of M₃-selective antagonists. Non-micturition activity has been described in normal human subjects and in patients with OAB syndrome. Thus, we can make the tentative suggestion that the anticholinergic drugs exert their therapeutic action by inhibiting the pacemaker activity in the bladder wall involved in the generation of this motor/sensory system.

FIG. 12. A schematic diagram suggesting a possible arrangement of mechanisms to account for the complex actions of carbachol and arecaidine and the muscarinic antagonists on the isolated whole bladder preparation. **A**, a block diagrammatic representation of the basic phenomena. Activation of the bladder via postganglionic parasympathetic nerves during voiding are shown. The different components of the phasic activity are shown: the initial fast component and the steady-state component. **B**, is a cartoon suggesting how these phenomena might be related to cellular mechanisms and anatomical features within the bladder. The possibility is suggested that there is: (i) A pacemaker element, driven by a mechanism dominated by M₃ receptors. (ii) A distribution network, involving M₂ components. This network contacts and communicates with the muscle resulting in phasic contractile activity. In the absence of muscarinic inputs phasic activity can be activated by the nicotinic ligand lobeline suggesting a further specialized region within the network. (iii) Contractions of the detrusor, controlled by M₃ receptors possibly activated directly by efferent parasympathetic cholinergic nerves.



ACKNOWLEDGEMENTS

We would like to acknowledge the support of Novartis for providing a contribution to the

resources needed to carry out this study and to Ms Hazel Rowntree for technical assistance in the preparation of these experiments.

CONFLICT OF INTEREST

None declared.

REFERENCES

- Abrams P, Cardozo L, Fall M *et al*. The standardisation of terminology of lower urinary tract function: report from the Standardisation Sub-Committee of the International Continence Society. *Neurourol Urodyn* 2002; **21**: 167–78
- Milsom I, Abrams P, Cardozo L, Roberts RG, Thuroff J, Wein AJ. How widespread are the symptoms of an overactive bladder and how are they managed? A population-based prevalence study. *BJU Int* 2001; **87**: 760–6
- Stewart WF, Herzog A, Wein A *et al*. Prevalence and impact of overactive bladder in the US: results from the NOBLE program. *Neurol Urodyn* 2001; **20**: 406–8
- Hashim H, Abrams P. Do symptoms of overactive bladder predict urodynamics detrusor overactivity? *Neurourol Urodyn* 2004; **23**: 484–6
- Andersson KE, Wein AJ. Pharmacology of the lower urinary tract: basis for current and future treatments of urinary incontinence. *Pharmacol Rev* 2004; **56**: 581–631
- Andersson KE, Yoshida M. Antimuscarinics and the overactive detrusor – which is the main mechanism of action? *Eur Urol* 2003; **43**: 1–5
- Finney SM, Andersson KE, Gillespie JI, Stewart LH. Antimuscarinic drugs in detrusor overactivity and the overactive bladder (OAB) syndrome: motor or sensory actions? *BJU Int* 2006; **98**: 503–7
- Andersson KE. Bladder activation: afferent mechanisms. *Urology* 2002; **59** (Suppl. 1): 43–50
- Kim Y, Yoshimura N, Masuda H, de Miguel F, Chancellor MB. Antimuscarinic agents exhibit local inhibitory effects on muscarinic receptors in bladder afferent pathways. *Urology* 2005; **65**: 238–42
- Yokoyama O, Yusup A, Miwa Y, Oyama N, Aoki Y, Akino H. Effects of tolterodine on an overactive bladder depends on suppression of C-fiber bladder afferent activity in rats. *J Urol* 2005; **174**: 2032–6
- Drake MJ, Harvey IJ, Gillespie JI. Autonomous activity in the isolated guinea pig bladder. *Exp Physiol* 2003; **88**: 19–30
- Gillespie JI, Harvey IJ, Drake MJ. Agonist and nerve-induced phasic activity in the isolated whole bladder of the guinea pig: evidence for two types bladder activity. *Exp Physiol* 2003; **88**: 343–57
- Gillespie JI. The autonomous bladder: a view of the origin of bladder overactivity and sensory urge. *BJU Int* 2004; **93**: 478–83
- Gillespie JI. A developing view of the origins of urgency: the importance of animal models. *BJU Int* 2005; **96** (Suppl. 1): 22–8
- Kashihara K, Varga EV, Waite SL, Roeske WR, Yamamura HI. Cloning of the rat M₃, M₄ and M₅ muscarinic acetylcholine receptor genes by the polymerase chain reaction (PCR) and pharmacological characterization of the expressed genes. *Life Sci* 1992; **51**: 955–71
- Andersson KE, Arner A. Urinary bladder contraction and relaxation: physiology and pathophysiology. *Physiol Rev* 2004; **84**: 935–86
- Yamanishi T, Chapple CR, Chess-Williams R. Which muscarinic receptor is important in the bladder? *World J Urol* 2001; **19**: 299–306
- Yamanishi T, Chapple CR, Yasuda K, Chess-Williams R. The role of M₂ muscarinic receptor subtypes in mediating contraction of the pig bladder base after cyclic adenosine monophosphate elevation and/or selective M₃ inactivation. *J Urol* 2002; **167**: 397–401
- Hegde SS, Choppin A, Bonhaus D *et al*. Functional role of M₂ and M₃ muscarinic receptors in the urinary bladder of rats *in vitro* and *in vivo*. *Br J Pharmacol* 1997; **120**: 1409–18
- Braverman AS, Karlovsky M, Pontari MA, Ruggieri MR. Aging and hypertrophy change the muscarinic receptor sub-type mediating contraction from M₃ to M₂. *J Urol* 2002; **167**: 43 (abstract 170)
- Chess-Williams R. Potential therapeutic targets for the treatment of detrusor overactivity. *Expert Opin Ther Targets* 2004; **8**: 95–106
- Barlow RB, Shepherd MK. A further search for selective antagonists at M₂-muscarinic receptors. *Br J Pharmacol* 1986; **89**: 837–43
- Mosso MA, Pellacani P. Sur les fonctions de la vessie. *Arch Ital Biol* 1882; **1**: 291–324
- Sherrington CS. Notes on the arrangement of some motor fibres in the lumbo-sacral plexus. *J Physiol* 1892; **13**: 621–772
- Vaughan CW, Satchell PM. Urine storage mechanisms. *Prog Neurobiol* 1995; **46**: 215–37
- Streng T, Hedlund P, Talo A, Andersson KE, Gillespie JI. Phasic non-micturition contractions in the bladder of the anaesthetized and awake rat. *BJU Int* 2006; **97**: 1094–101
- Starling EH. *Elements of Human Physiology*, 7th edn. London: Churchill, 1905
- Gillespie JI. Modulation of autonomous contractile activity in the isolated bladder of the guinea pig. *BJU Int* 2004; **93**: 393–400
- Gillespie JI. Inhibitory actions of calcitonin gene-related peptide and capsaicin: evidence for local axonal reflexes in the bladder wall. *BJU Int* 2005; **95**: 149–56
- Gillespie JI. Noradrenaline inhibits autonomous activity in the isolated guinea pig bladder. *BJU Int* 2004; **93**: 401–9
- Hammer R, Giraldo E, Schiavi GB, Monferini E, Ladinski H. Binding profile of a novel cardioselective muscarinic receptor antagonist AF-DX 116, to membranes of peripheral tissues and brain in the rat. *Life Sci* 1986; **38**: 1653–62
- Barlow RB, Weston-Smith P. The relative potencies of some agonists at M₂ muscarinic receptors in guinea-pig ileum, atria and bronchi. *Br J Pharmacol* 1985; **85**: 437–40
- Gillespie JI. Phosphodiesterase-linked inhibition of nonmicturition activity in the isolated bladder. *BJU Int* 2004; **93**: 1325–32
- Smet PJ, Jonavicius J, Marshall VR, de Vente J. Distribution of nitric oxide synthase-immunoreactive nerves and identification of the cellular targets of nitric oxide in guinea-pig and human urinary bladder by cGMP immunohistochemistry. *Neuroscience* 1996; **71**: 337–48
- McCloskey KD, Gurney AM. Kit positive cells in the guinea pig bladder. *J Urol* 2002; **168**: 832–6
- Hashitani H, Yanai Y, Suzuki H. Role of

- interstitial cells and gap junctions in the transmission of spontaneous Ca^{2+} signals in detrusor smooth muscles of the guinea-pig urinary bladder. *J Physiol* 2004; **559**: 567–81
- 37 Gillespie JI, Markerink-van Ittersum M, de Vente J. cGMP-generating cells in the bladder wall: identification of distinct networks of interstitial cells. *BJU Int* 2004; **94**: 1114–24
- 38 Davidson RA, McCloskey KD. Morphology and localization of interstitial cells in the guinea pig bladder: structural relationships with smooth muscle and neurons. *J Urol* 2005; **173**: 1385–90
- 39 Gillespie JI, Markerink-van Ittersum M, de Vente J. Interstitial cells and cholinergic signalling in the outer muscle layers of the guinea-pig bladder. *BJU Int* 2006; **97**: 379–85
- 40 Lagou M, Drake MJ, Markerink-van Ittersum M, de Vente J, Gillespie JI. Interstitial cells and phasic activity in the isolated mouse bladder. *BJU Int* 2006; **98**: 643–50
- 41 Gillespie JI, Markerink-van Ittersum M, de Vente J. Sensory collaterals, intramural ganglia and motor nerves in the guinea-pig bladder: evidence for intramural neural circuits. *Cell Tissue Res* 2006; **325**: 33–45

Correspondence: James I. Gillespie, The Urophysiology Research Group, School of Surgical and Reproductive Sciences, The Medical School, The University, Newcastle upon Tyne, NE2 4HH, UK.
e-mail: j.i.gillespie@ncl.ac.uk

Abbreviations: OAB, overactive bladder; DO, detrusor overactivity; 4-DAMP, 4-diphenylacetoxy-N-methylpiperidine methobromide; F_{init} , frequency of intravesicle pressure transient rises in the initial phase after agonist application; F_{ss} , frequency of transients in the steady state; IC, interstitial cell; ChAT, choline acetyl transferase; NF, neurofilament.

Volume-induced responses in the isolated bladder: evidence for excitatory and inhibitory elements

Steven M. Finney, Laurence H. Stewart and James I. Gillespie*

*Western General Hospital, Edinburgh, Scotland, and *The Urophysiology Research Group, Institute for Cellular Medicine, The University, Newcastle upon Tyne, UK*

Accepted for publication 10 April 2008

OBJECTIVE

To quantify changes in autonomous activity in response to alterations in intravesical volume, to explore the possible underlying regulatory mechanisms.

MATERIALS AND METHODS

Experiments were conducted using whole isolated bladders from six female guinea pigs (280–400 g). A cannula was inserted into the urethra to monitor intravesical pressure and the bladder was suspended in a heated chamber containing carboxygenated physiological solution at 33–36 °C. All drugs were added to the solution on the abluminal bladder surface.

RESULTS

An increase in intravesical volume followed by a rapid reduction lead to a complex series of activity comprising of several distinct phases. After a volume increase there was an initial 'burst' of frequency which gradually declined to a 'steady state'. After a volume reduction there was a period of quiescence

with spontaneous activity gradually returning to levels seen before the increase, termed the 'inhibitory phase'. The frequency of transient contractions, both immediately after a volume increase and at steady state, increased both with increasing intravesical volume and dose of arecaidine. The length of the inhibitory phase increased both with the duration and magnitude of volume increase. However, the inhibitory phase was not entirely dependent on the magnitude of volume change, as the inhibitory phase was shorter when the volume was not returned to baseline levels. Increasing doses of arecaidine shortened the inhibitory phase.

CONCLUSIONS

These observations suggest that the regulation of volume-induced spontaneous activity relies on complex excitatory and inhibitory inputs. The rapid burst of activity resulting from a rise in volume suggests the presence of a rapidly adapting mechanism. Rapid reduction in intravesical volume leads to a quiescent period, i.e. the inhibitory phase. This is related to both the duration of intravesical volume increase and its

magnitude. However, similar volume changes are more effective when the volume is reduced back to baseline, as opposed to the bladder being incompletely emptied. Furthermore, the frequency of transient contractions remained constant once a steady state was reached, with no evidence of inhibition before volume reduction. This suggests that mechanisms involved in the generation of the inhibitory phase initiated during bladder filling require >30 s to have a significant effect, but depend on a reduction in volume to be triggered, with the response dependent on the volume reduced. The mechanisms involved in generating and modulating the inhibitory phase seem to be regulated by a strong cholinergic input but the exact nature of these mechanisms is unknown. The potential importance of these results in terms of the general physiology and pharmacology of the bladder is discussed.

KEYWORDS

autonomous activity, volume-induced activity, inhibitory phase

INTRODUCTION

The isolated bladder is capable of generating spontaneous co-ordinated activity involving localized contractions and propagating waves over the bladder surface. This activity, termed 'autonomous activity', results in an increase in intravesical pressure consisting of regular, rhythmical transient contractions [1,2]. This activity can be augmented by adding the muscarinic agonists arecaidine or carbachol to give larger increases in intravesical pressure, associated with increases in the

amplitude of localized contractions and frequency of the propagating waves [2]. Autonomous activity has been studied most extensively in the guinea pig, but has also been reported in other animals [3–5]. Similar activity analogous to autonomous activity, unrelated to the act of micturition, has also been shown to be present during the filling phase of many species, including man [3,6–10]. In the guinea pig, autonomous activity augmented by muscarinic stimulation can be manipulated in several ways. The frequency of transient contractions can be

increased by adding α,β -methylene ATP (a nonhydrolysable analogue of ATP), substance P and sodium nitroprusside (a nitric oxide donor) [11,12], whereas there is a reduction in frequency with noradrenaline [13]. These combined findings suggest that the mechanisms regulating phasic activity receive both excitatory and inhibitory inputs [12,13].

The physiological role of autonomous activity is not understood. Autonomous activity and augmented activity are associated with localized microcontractions (micromotions)

of the bladder wall, accompanied by local stretches [1–4]. Such activity occurs in both the normal and pathological bladder, where local contractions generate small pressure changes accompanied by local stretches [1–3]. This phasic motor activity is associated with phasic discharges in afferent nerves [14–16], suggesting that it forms the motor component of a motor/sensory system in the bladder wall.

This idea that such a motor/sensory system existed in the bladder was first proposed in the early part of the 20th century [14]. It has also been known for some time that this motor/sensory system has the capacity to be modulated by different inputs. Data taken from cat experiments show that the amplitude and frequency of this activity can be influenced by sympathetic nerve inputs [6,8].

In response to increasing intravesical volume the frequency and amplitude of transient contractions has been found to increase [17]. As phasic activity is considered to be part of an integrated motor/sensory system, given its responses it is suggested that the system receives inputs directly related to intravesical volume. It was previously proposed that several complex arrangements exist within the bladder that could account for the physiological responses seen within phasic and volume-related activity. The excitatory effect of substance P and the inhibitory effect of calcitonin gene-related peptide (CGRP) on phasic activity suggest the presence of a sensory-axon collateral reflex within the bladder wall, supporting a role for the release of sensory neurotransmitters within the wall of the bladder [18]. It was documented previously that afferent nerves can send collateral fibres to structures within the tissue where they originate [19–21]. However, with prolonged exposure to capsaicin, leading to the elimination of substance P/CGRP sensory nerves [20], volume-induced changes are not affected. This suggests that the local sensory-collateral reflex is not involved in the volume response. Also, as all potential neural sensory influences would be removed via the action of capsaicin, non-neural mechanisms must therefore be involved in the regulation of volume-induced activity.

In addition to the effects of increasing intravesical volume, a rapid decrease results in a period of quiescence, where there is a prolonged period of inhibition of phasic

activity, followed by a gradual return [17]. The mechanisms involved in this process are unknown, although given the lack of effects of tetrodotoxin or capsaicin they are likely to be strongly related to non-neuronal activity (as described previously). However, the actions of ODO, an inhibitor of guanyl cyclase, suggest a potential role for interstitial cells in either its generation or co-ordination [17]. Other potential non-neuronal sources of inhibition have been proposed, one of which is a urothelium-derived 'inhibitory factor' able to inhibit contraction of detrusor smooth muscle [8], although its effects upon phasic activity have not been assessed.

Changes in phasic activity in response to changes in intravesical volume are complex. It is proposed that phasic activity is related to sensation and therefore might be a mechanism through which bladder volume can be assessed. Inhibition after a volume decrease might occur to suppress sensation during or immediately after voiding that could otherwise be uncomfortable. The mechanisms behind these volume-related responses are unknown, although they seem to be related to local mechanisms inherent to the bladder wall. The aim of the present experiments was to further characterize the intrinsic properties of these responses in an attempt to further illustrate potential mechanisms through which they might act, and assess their role in the integrative physiology of the isolated bladder.

MATERIALS AND METHODS

The procedures for isolating the whole bladder and maintaining it *in vitro* were described previously [1,2]. Briefly, 13 female guinea pigs (weight 280–400 g) were killed by cervical dislocation in accordance with schedule 1 of the UK Home Office regulations. The urinary bladder and urethra were removed and placed in Tyrode's solution (composition, mM: NaCl, 120; KCl 4.5; CaCl₂ 2.5; MgCl₂ 1; NaHCO₃ 25; NaH₂PO₄ 1; Na pyruvate 1, glucose 5) bubbled with 5% CO₂ and 95% O₂ (pH 7.4). The mean (SD) bladder weight was 303 (55) mg (11 guinea pigs). A flexible plastic cannula (2 mm diameter) was inserted into the urethra and secured at the bladder neck using a thread ligature. Residual urine was removed and replaced with Tyrode's solution. The bladder was transferred to a heated organ bath (30 mL, 33–36 °C) containing constantly gassed Tyrode's

solution. The cannula was connected with the urethra uppermost via a fluid-filled tube and three-way connector to a pressure transducer (DTX Plus, Becton Dickinson, Franklin Lakes, NJ, USA) and a 2.5-mL syringe to enable the intravesical volume to be varied. The transducer output was amplified, digitized (sampling rate 10 Hz) and recorded using an computer-based capture system. The transducer was calibrated before each experiment. At the outset each bladder was filled with Tyrode's solution to give a baseline volume of 600–800 μ L. These volumes were typically the amount of urine recovered from guinea pigs immediately after cervical dislocation. Volumes were changed typically over 10 s for each specific increase/decrease. The time taken from killing the guinea pig to beginning recording was typically 30 min. Pressure recording was begun immediately but the bladder was left to equilibrate for ≥ 20 min before any manipulations. The drug used was the muscarinic agonist arecaidine but-2-ynyl ester tosylate (Tocris, UK). Concentrated drug solutions were added directly to the bath to achieve the required final dilution. All drugs were added to the solution bathing the serosal surface of the isolated whole bladder.

Quantitative values are given as the mean (SD), with the significance of differences between means determined using a paired *t*-test, the level for significance being set at 0.05. The number of preparations used in the statistical analysis for each experiment and each condition are given in the appropriate figure legends.

RESULTS

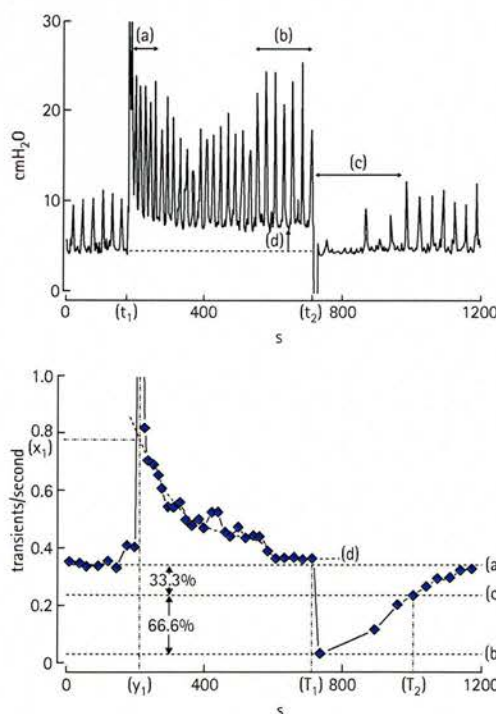
For each of the experiments a standard control response was obtained [17], and the method underpinning the analysis of the experimental traces is shown in Fig. 1. The effects of the magnitude of intravesical volume change, duration of volume increase and increasing doses of arecaidine were all assessed individually. To assess the effects of the magnitude of volume change, experiments were conducted in the presence of 100 nM arecaidine. Volumes were increased for 500 s before a decrease. Typical examples of the responses from the same organ preparation are shown in Fig. 2. The frequency of transient contractions, both immediately after a volume increase (IF_{init}) and at steady state (F_{ss}), were higher after larger volume increases. Furthermore, IF_{init} represents a

'burst' of activity that slowly reduced to form a 'steady state'. Also, the length of the inhibitory phase was not solely dependent on the increase in intravesical volume. Figure 2a,b show that the length of the inhibitory phase was related to the magnitude of volume increase if the volume was then reduced back to baseline values. However, Fig. 2c highlights that despite a similar volume change, compared to that in Fig. 2b, the inhibitory phase was reduced if the volume was not fully returned to baseline. This suggests that both volume increase and decrease are important factors in generating the inhibitory phase. Further comparison of the traces also shows that the shift in underlying pressure (P_{shin}) also increased at higher volumes. Figure 3 shows an analysis of these results obtained from five separate guinea pigs.

The effects of a variable duration of filling were assessed by increasing the volume for 10, 30, 100 and 500 s, respectively. All responses were assessed in the presence of 100 nM arecaidine and by increasing/decreasing the volume by 2000 μL . A typical example of the differing duration of increase, applied to the same isolated bladder, is shown in Fig. 4. As the volumes both at baseline and after an increase remain constant there were no differences in the frequency of either the pre-filling or initial phase between volume responses. What is apparent is that the inhibitory phase increased with the duration of volume increase. Figure 5 shows an analysis of the inhibitory phase reported from five separate bladders, again showing that the inhibitory phase was related to the duration of the volume increase. There was a statistically significant difference between all data points apart from the durations 10 and 30 s.

The effects of the two arecaidine concentrations (100 and 300 nM) on volume-induced responses were compared, with typical examples from the same bladder shown in Fig. 6. All volume responses were assessed with a volume change of 2000 μL and duration of fill of 500 s. It was described previously that increasing the dose of arecaidine results in an overall increase in the frequency and amplitude of transient contractions [2]. However, these experiments also showed that increasing doses of arecaidine also increased IF_{init} in addition to F_{ss} . The effects were similar on the increase in P_{shin} , with this being larger at higher doses.

FIG. 1. Top panel; typical example of a 'volume response' obtained from an isolated guinea pig bladder, augmented by 100 nM arecaidine. t_1 represents the time at which intravesical volume was increased and t_2 the point where volume was reduced back to baseline volume. ($t_1 - t_2$) is termed the 'duration of volume increase'. The interval increasing, or decreasing, volume is typically <10 s. (a) period immediately after a volume increase, where there is an initial increase in frequency and underlying pressure, followed by a gradual decrease. Qualitatively this area immediately after the volume increase is termed the 'initial phase'. (b) period after a volume increase where the frequency, amplitude and underlying pressure of transient contractions remain relatively constant, termed 'steady state'. (c) time immediately after a volume decrease, where there is a quiescent period followed by a gradual return in activity, termed the 'inhibitory phase'. A clear demarcation of the end of the inhibitory phase is difficult; therefore, the point at which the frequency returns to 66.6% of the frequency before the volume increase was typically used in calculations; labelled the ' τ '. (d) refers to the points used to calculate the increase in underlying pressure. Bottom panel; illustrates the change in instantaneous frequency of the transients during the volume response shown above. The red dashed line represents a line of best fit drawn during the period of increased volume. (y_1) represents a vertical axis drawn at the point of volume increase. Intersection of the line of best fit and (y_1) represents the frequency at the point of volume increase (IF_{init}), extrapolation of this intersection to the ordinate axis giving the value of IF_{init} shown as (x_1). In this case IF_{init} equals 0.78 transients/s. (d) represents the F_{ss} . (a-c) are used in the calculation of τ for the inhibitory phase, the point when frequency returns to 66.6% of its baseline 'before volume increase'; (a) represents the baseline frequency before the volume increase - note the frequency after a volume decrease is returning to this level; (b) represents the lowest frequency after a volume decrease; (c) represents an arbitrary line drawn 66.6% of the distance between (a) and (b). (T_1) represents the time at volume decrease (T_2) is extrapolated from the intersection between the instantaneous frequency plot and (c); representing the point at which the frequency returns to 66.6% of baseline, i.e. τ for the inhibitory phase = ($T_2 - T_1$).



Conversely, the inhibitory phase was significantly shorter in the presence of the higher dose of arecaidine. Figure 7 shows mean data collected from five different

bladders on the effects of 100 and 300 nM arecaidine on the inhibitory phase, change in underlying pressure at steady state (P_{shin}), IF_{init} and F_{ss} (Fig. 7a-d), respectively.

FIG. 2. Three volume responses, relating to differing volume changes, from the same isolated guinea pig bladder preparation. The amount by which intravesical volume was increased and then decreased is illustrated beneath each trace. Each response was assessed in the presence of 100 nM arecaidine and the volume increased for 500 s: (a) a 2.0-mL increase followed by a 2.0-mL decrease; (b) a 1.5-mL increase and 1.5 mL decrease; and (c) a 2.0-mL volume increase with a decrease of 1.5 mL. Ordinate axes, pressure (cmH₂O) and abscissa time (s). The pre-filling phases are similar for all three, but in comparing (a) and (b) there is an increase in frequency initially and at steady state. Also, note a reduction in the inhibitory phase from (a) through to (c).

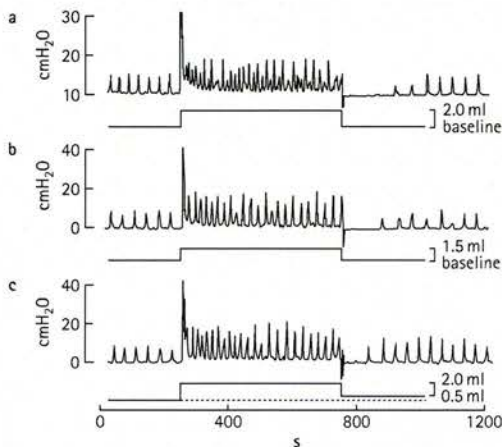


FIG. 4. Volume responses with a varying duration of intravesical volume increase, assessed in the presence of 100 nM arecaidine with volume changes of 2.0 mL. The duration of each volume increase is shown beneath each trace. Ordinate, pressure (cmH₂O) and abscissa, time (s).

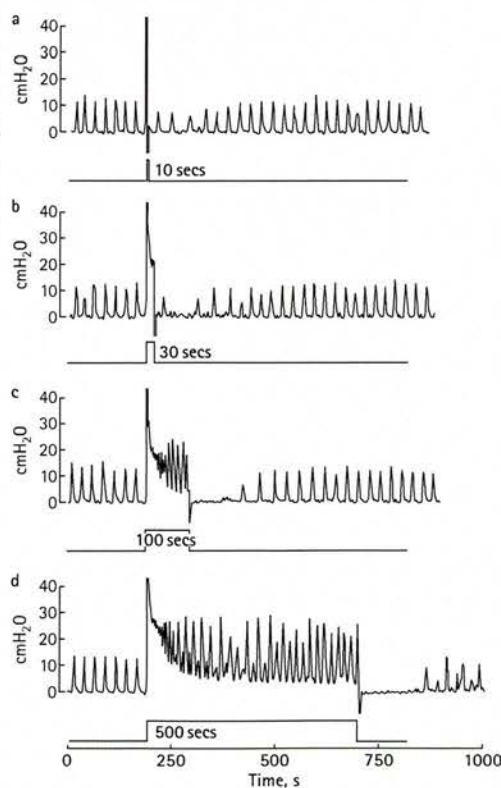
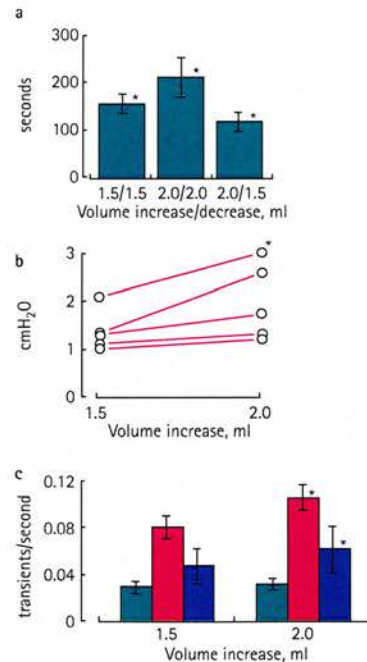


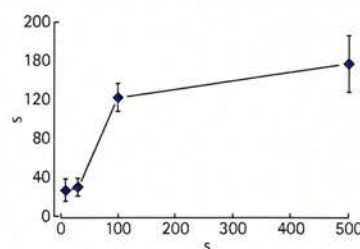
FIG. 3. Data accumulated from five bladders illustrating the effects of different volume changes on the volume response: (a) the effects of three combinations of volume changes on τ of the inhibitory phase. Ordinate, τ (s) and abscissa, volume increase/decrease (mL); (b) represents P_{shift} . Ordinate, pressure (cmH₂O) and abscissa, volume increase (mL); (c) the effects of two volume increases on IF_{mi} (maroon), and at F_{ss} (yellow). The blue column represents the baseline transient frequency before the volume increase. Ordinates, frequency (transients/s) and abscissae, volume increase (mL). Data points separated by a line in (b) represent results from the same bladder. Data in (a) + (c) are shown as the mean (SD). A significant difference between volume changes is indicated (* $P < 0.05$, Student's *t*-test).



DISCUSSION

It was shown previously that changes in intravesical volume lead to changes both in the frequency and amplitude of spontaneous phasic activity [1,2], and that spontaneous transient contractions were associated with a burst of afferent discharge. As phasic activity is considered to be part of an integrated motor/sensory system, the responses in activity to volume suggest that the CNS receives inputs directly related to intravesical volume. In addition, it was also shown that

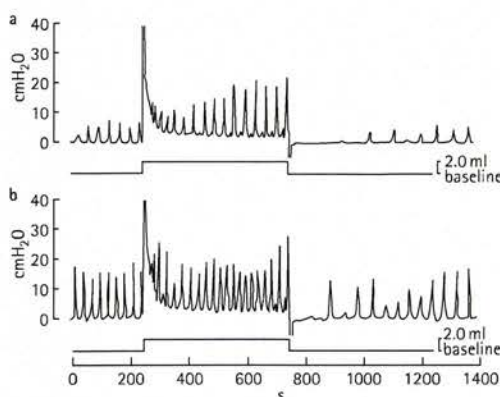
FIG. 5. Data acquired from five bladders showing the effects of different duration of volume increase on τ of the inhibitory phase (s) and abscissae, duration of volume increase (s). Data points are shown as the mean (SD). Apart from at 10–30 s, there was a significant difference between all other values ($P < 0.05$, Student's *t*-test).



phasic activity can receive further excitatory and inhibitory inputs [11,12], and as such afferent discharge related to volume could be influenced by these other stimuli. Therefore, sensory perception of intravesical volume has the capacity to be modulated by mechanisms acting in the periphery [17]. However, there are several major unanswered questions relating to the generation and modulation of the phasic activity, and to the specific cellular mechanisms associated with the volume-induced changes in activity.

The urothelium is known to release ATP in response to mechanical distortion [8]. It was suggested that the role of urothelial-released ATP is to modulate the firing of afferent nerve fibres, in turn influencing bladder sensation [22–24]. However, a further suggestion is that ATP can indirectly act on the bladder via alterations in phasic activity [10], inducing sensory neurotransmitter release via antidromic activation of collateral afferent nerve fibres [17]. However, the effect on volume-induced inhibition has not been assessed. Further potential sources of non-neuronal mechanisms might include interstitial cells and a nonspecific substance termed 'inhibitory factor'. Interstitial cells could have a role in modulating volume-induced activity by inhibiting phasic activity after a volume reduction, as ODQ, an inhibitor of guanylate cyclase, reduced the duration of this inhibition [17], whilst 'inhibitory factor' is speculated to be released from the urothelium in response to stretch that results in an inhibition of detrusor contraction [25]. However, the results on phasic activity again have not been assessed.

FIG. 6. Typical traces of volume responses assessed in the presence of 100 and 300 nM arecaidine, (a) and (b), respectively. The amounts by which the intravesical volumes were changed are shown beneath each trace, in addition to the duration of volume increase. In these cases volume changes were 2.0 mL, lasting for 500 s. Ordinates represent pressure (cmH₂O) and the abscissa, time (s). Note the increased frequency in all three phases (before filling, initial, steady state) in the presence of 300 nM arecaidine (b), compared to 100 nM (a). Also, note the reduction in the inhibitory phase in the presence of 300 nM arecaidine (b).



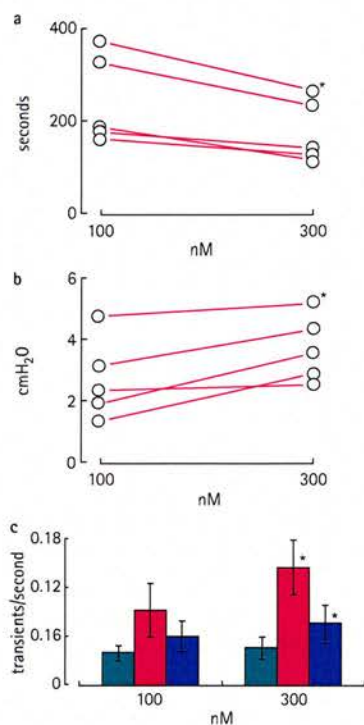
A pacemaker mechanism appears to be a key component generating phasic activity [26]. Therefore, inputs from various sources must integrate here to exert their effect. The location and nature of this potential pacemaker mechanism are unknown. In the guinea pig, a recent study showed the presence of a suburothelial ganglionic network, the neurones of which are contacted by different types of nerve fibre. Three types were identified: fibres expressing CGRP, fibres expressing choline acetyltransferase (ChAT) and neurofilament protein (ChAT/NF⁺), and fibres expressing ChAT but not neurofilament (ChAT/NF⁻) [27]. The CGRP and ChAT/NF⁻ fibres might be collaterals of sensory fibres that appear to originate in the suburothelial layer. The ChAT/NF⁺ fibres might represent axons from adjacent ganglia. Furthermore, two types of nerve fibres are known to innervate the muscle layer, i.e. ChAT/NF⁺ and ChAT/NF⁻ fibres. The ChAT/NF⁺ fibres might also contact interstitial cells in the outer muscle layer. Thus, there is evidence for a suburothelial ganglionic network with the potential to receive inputs from the urothelium and adjacent ganglia, which has an output to the muscle layers. It was speculated that such a network is involved in the local bladder wall reflexes linking urothelial distortion to phasic contractile activity [27]. The suburothelial ganglion cells receive inputs from two types of nerve fibre that are likely to be cholinergic, suggesting

that the ganglionic cells express cholinergic receptors. Therefore, the mediation of ganglionic activity is likely to have a strong cholinergic influence, with application of exogenous muscarinic agonists activating these ganglia. Thus, there is circumstantial evidence to support a structural framework that has the potential to mediate the responses in phasic activity previously described, in addition to allowing speculation as to their involvement in volume-induced changes.

The present results illustrate the responses of the isolated guinea pig bladder to changes in intravesical volume, duration of volume increase and the effects of the two doses of arecaidine (100 and 300 nM) on volume-induced 'augmented' activity ('volume response'). After an increase in intravesical volume there is a complex series of events, similar to those described after adding arecaidine [26]; again an 'initial phase' consisting of a burst of high-frequency, low-amplitude transients followed by a 'steady state' consisting of regular transient contractions of a lower frequency but greater amplitude. After a reduction in intravesical volume there is a period of quiescence, with the transient contractions gradually returning to their baseline frequency and amplitude.

The 'initial phase' after a volume increase comprises a succession of rapid, small-

FIG. 7. Data collected from five different isolated bladder preparations on the effects on the volume response in the presence of 100 and 300 nM arecaidine. Data points connected by a line represent results collected from the same bladder preparation; (a) the effects of the two arecaidine doses on the inhibitory phase; ordinate axis, τ for the inhibitory phase (s); (b) represents the effects on P_{shift} ; ordinate axis, pressure (cmH₂O); (c) the effects on frequency after a volume increase (IF_{init} , (maroon) and F_{ss} , (yellow). The blue column represents the baseline frequency before any volume increase. Data are shown as the mean (SD). Ordinate axis, instantaneous frequency of transients (transients/s). In all three panels the abscissae relate to the two doses of arecaidine in which the volume response was assessed. There was a significant difference between the effects of the two concentrations, * $P < 0.05$, Student's *t*-test.



amplitude transient contractions superimposed on an initial sudden increase in underlying basal pressure, followed by a gradual decline. The overall magnitude of this 'initial' response depends on the increase in intravesical volume, as illustrated by both IF_{init} and P_{shift} , being significantly higher after a 2-mL than a 1.5-mL increase. However, as P_{shift} declines to 'steady state' levels, frequency also

gradually reduces, with the amplitude of transients increasing. As the detrusor is composed of slowly adapting smooth muscle the decrease in P_{shift} after a volume increase could be accounted for by relaxation of the detrusor as it accommodates the increased volume. However, as the urine within the bladder will act as any other fluid, being relatively incompressible, the volume within the bladder would remain relatively constant despite the pressure change. As the frequency of phasic activity is related to intravesical volume the decrease in frequency after a volume increase suggests the governing mechanisms become 'overloaded', requiring a period of adaptation before the true volume-related frequency is achieved (steady state). Although these volumes have been increased at unphysiological rates the findings suggest the mechanisms involved in the regulation of volume-related frequency have a component that is rapidly adapting, allowing a degree of desensitization to occur in response to high rates of filling.

The magnitude of the 'initial phase' seems to be directly related to the magnitude of the increase in intravesical volume, as shown by the differences between increases of 2 and 1.5 mL. Also, when the raw data were analysed from previous records illustrating the effects of smaller sequential increases in volume on the initial phase, the initial phase was smaller [17]. However, the final 'steady state' between the present data and that of Lagou *et al.* [17] at comparable volumes, remains similar, suggesting that the 'steady state' is unaffected by the rate of volume increase. Furthermore, under physiological conditions there is no 'initial phase', although a gradual increase in frequency, amplitude and underlying pressure is apparent [28], similar to that seen in the steady state at increasing volumes during the present experiments. Also, on detailed inspection of these records an inhibitory phase is also evident, illustrating that quiescence after a volume reduction is a true physiological response, as opposed to a factor related to increased filling rates [28].

After a volume reduction there is a period of quiescence, termed the 'inhibitory phase'. As noted previously, this was apparent both *in vitro* [17] and *in vivo* [28]. The present experiments further illustrate the 'inhibitory phase' to be influenced by several factors, consisting of volume increase, duration of increase, muscarinic agonist concentration

and degree of bladder emptying. The length of the inhibitory phase increased with volume, but this was not entirely dependent on the absolute change in volume. It was shown that the length of the inhibitory phase also depends on the volume decrease, with longer inhibitory phases when the volume returned to baseline. This is highlighted when the mean results of a volume increase and decrease of 1.5 mL (157 s) are compared with those after a volume increase of 2.0 mL and decrease of 1.5 mL (118 s). From this simple observation it could be argued that a two-stage process is evident in the regulation of the inhibitory phase. A volume increase might act as a volume-dependent stimulus for the synthesis of agents, or initiation of mechanisms, involved in the inhibitory phase that are released/triggered again in a volume-dependent manner as the bladder empties. In turn, this suggests that the stimulus of volume decrease is of similar importance to that of volume increase in determining the length of the inhibitory phase. It can be further argued that an increase in the inhibitory phase after a return to baseline volume would suggest that maximum mechanical distortion, as opposed to pure stretch, is required for the full effects of the inhibitory response to be seen. Whether this is predominantly related to specific structures is open to speculation, but it might indicate a role for the urothelium, as this might be indicative of the urothelium unfolding as the bladder fills and re-folding as it empties. ATP and nitric oxide released from the urothelium on unfolding/re-folding could easily have an effect on suburothelial purinergic receptors [22–24] or interstitial cells [17]. Certainly, suburothelial interstitial cells seem to be implicated in some way to the inhibitory phase, as ODO, an inhibitor of guanylate cyclase and therefore cell signalling via cGMP, i.e. interstitial cells, significantly reduces its length.

The duration of volume increase also has a role in the length of the inhibitory phase, with a longer duration of filling being associated with longer inhibitory phases. There was an insignificant difference in the length of the inhibitory phase after a duration of filling of <30 s, with a small difference between the duration of fill of 100 and 500 s. However, there was a substantial difference after a 30- and 100-s volume increase, suggesting that the rate of increase in the mechanisms involved in the regulation of the inhibitory phase increases substantially between these

time points. This relationship between the inhibitory phase and duration of filling further supports the idea of agents/mechanisms involved in the inhibitory phase being synthesized/initiated in response to a volume increase. It also supports the inhibitory phase to be a true physiological response as opposed to flaccidity related to stretch-induced damage of the underlying detrusor muscle.

The response of phasic activity in the isolated bladder to alterations in intravesical volume is complex. However, the effects illustrated after adding 100 and 300 nM arecaidine suggest a strong cholinergic component, or components, involved in the modulation of all three stages; initial, steady state and inhibitory phase, with cholinergic inputs exerting an excitatory effect by acting directly on the pacemaker mechanisms. This increased pacemaker stimulus results in an increase in IF_{init} and F_{ss} . However, it also leads to a reduction in the inhibitory phase. This suggests that the mechanisms involved in generating the inhibitory phase involve a balance between both inhibitory and excitatory stimuli, with an excitatory component being cholinergically mediated.

The mechanisms involved in providing the inhibitory stimulus for the inhibitory phase are not entirely known. It was suggested previously that interstitial cells might play some part in modulating the inhibitory phase [17], although undoubtedly additional factors also need to be considered. The observations described previously add weight to the argument that distortion of the urothelium might release factors involved in inhibition, with maximal distortion and a duration of volume increase of >30 s being required for maximum inhibition. One of these factors includes ATP, another being that of 'inhibitory factor' previously described [25]. 'Inhibitory factor' is derived from the superficial layer of the bladder and has been shown to be released after mechanical distortion, leading to a decrease in the strength of detrusor contraction. It was also shown to be a readily diffusible substance, as it can also reduce contractions in nearby undistorted bladders. Certainly, in the present observations, there was a reduction in amplitude during the inhibitory phase, associated with a reduction in frequency. However, during the inhibitory phase, electrical-field stimulation results in detrusor contraction [17], suggesting that the inhibitory phase is not related to flaccidity of

the detrusor muscle. Furthermore, possibly the inhibitory, and other factors, involved in the inhibitory phase exert their effects on the control of the detrusor, as opposed to the detrusor itself.

From these observations it was speculated that the regulation of volume-induced spontaneous activity and modulation of the inhibitory phase relies on a complex series of excitatory and inhibitory inputs. A rapid increase in intravesical volume leads to a burst of increased frequency that gradually declines, suggesting the presence of a rapidly desensitizing mechanism involved in modulating volume-related activity that becomes pronounced at high rates of filling. A rapid reduction in intravesical volume leads to a quiescent period, the inhibitory phase. This is related to both the duration of intravesical volume increase and its magnitude. However, similar volume changes are more effective when volume is reduced back to baseline, as opposed to the bladder being incompletely emptied. Furthermore, the frequency of transient contractions remains constant once a steady state has been reached, with no evidence of inhibition before volume reduction. These functional observations suggest that mechanisms involved in generating the inhibitory phase initiate during bladder filling, requiring >30 s to exert a significant effect. However, they depend on a reduction in volume to be triggered, with the response dependent on the volume reduced. The mechanisms involved in generating and modulating the inhibitory phase seem to be regulated by a strong cholinergic input, but the exact nature of these mechanisms is unknown. Certainly structures have been identified, in the form of interstitial cells and ganglia, capable of generating and modulating such activity. Furthermore, their close proximity to the urothelium which is capable of releasing ATP, nitric oxide and acetylcholine in response to stretch, suggests a potential role for the urothelium in the transduction of such activity.

CONFLICT OF INTEREST

None declared.

REFERENCES

- 1 Drake MJ, Harvey IJ, Gillespie JI. Autonomous activity in the isolated guinea pig bladder. *Exp Physiol* 2003; **88**: 19–30
- 2 Gillespie JI, Harvey IJ, Drake MJ. Agonist and nerve induced phasic activity in the isolated whole bladder of the guinea pig: evidence for two types of bladder activity. *Exp Physiol* 2003; **88**: 343–57
- 3 Sherrington CS. Notes on the arrangement of motor fibres in the lumbo-sacral plexus. *J Physiol* 1892; **13**: 621–772
- 4 Coolsaet BL, Van Duyl WA, Van Os-Bossagh P, De Bakker HV. New concepts in relation to urge and detrusor activity. *Neurol Urodyn* 1993; **12**: 463–71
- 5 Sugaya K, de Groat WC. Influence of temperature on activity of the isolated whole bladder preparation of neonatal and adult rats. *Am J Physiol Integr Comp Physiol* 2000; **278**: R238–46
- 6 Gjone R. Peripheral autonomic influence on the motility of the urinary bladder in the cat. I. Rhythmic contractions. *Acta Physiol Scand* 1965; **65**: 370–7
- 7 Plumb F, Colfelt RH. The genesis of vesical rhythmicity. *AMA Arch Neurol* 1960; **2**: 487–96
- 8 Vaughan CW, Satchell PM. Urine storage mechanisms. *Prog Neurobiol* 1995; **46**: 215–37
- 9 Bristow SE, Neal DE. Ambulatory urodynamics. *Br J Urol* 1996; **77**: 333–8
- 10 Gillespie JI. The autonomous bladder. A view of the origin of bladder overactivity. *BJU Int* 2004; **93**: 478–83
- 11 Gillespie JI. Modulation of autonomous contractile activity in the isolated bladder of the guinea pig. *BJU Int* 2004; **93**: 393–400
- 12 Gillespie JI, Drake MJ. The actions of sodium nitroprusside and the phosphodiesterase inhibitor dipyrindimole on phasic activity in the isolated guinea pig bladder. *BJU Int* 2004; **93**: 851–9
- 13 Gillespie JI. Noradrenaline inhibits autonomous activity in the isolated guinea pig bladder. *BJU Int* 2004; **93**: 401–9
- 14 Starling EH. *Elements of Human Physiology*, 7th edn. London: Churchill, 1905
- 15 Iggo A. Tension receptors in the stomach and urinary bladder. *J Physiol* 1955; **128**: 593–607
- 16 Shea VK, Cai R, Crepps B, Mason JL, Perl ER. Sensory fibers of the pelvic nerve innervating the rat's urinary bladder. *J Neurophysiol* 2000; **84**: 1924–33

- 17 Lagou M, Drake MJ, Gillespie JI. Volume-induced effects on the isolated bladder: a possible local reflex. *BJU Int* 2004; **94**: 1356–65
- 18 Gillespie JI. Inhibitory actions of calcitonin gene related peptide and capsaicin: evidence for local axonal reflexes in the bladder wall. *BJU Int* 2005; **95**: 149–56
- 19 Maggi CA. The dual function of capsaicin sensory nerves in the bladder and urethra. *Ciba Found Symp* 1990; **151**: 77–83
- 20 Maggi CA, Meli A. The sensory-efferent function of capsaicin sensitive sensory neurons. *Gen Pharmacol* 1988; **19**: 1–43
- 21 Maggi CA, Meli A. The role of neuropeptides in the regulation of the micturition reflex. *J Auton Pharmacol* 1986; **6**: 133–62
- 22 Ferguson DR, Kennedy I, Burton TJ. ATP is released from rabbit urinary bladder epithelial cells by hydrostatic pressure changes – a possible sensory mechanism? *J Physiol* 1997; **505**: 503–11
- 23 Birder LA, Apodaca G, De Groat WC, Kanai AJ. Adrenergic- and capsaicin-evoked nitric oxide release from urothelium and afferent nerves in urinary bladder. *Am J Physiol* 1998; **275**: F226–9
- 24 Lee HY, Bardini M, Burnstock G. Distribution of P2X receptors in the urinary bladder and the ureter of the rat. *J Urol* 2000; **163**: 2002–7
- 25 Hawthorn MH, Chapple CR, Cock M, Chess-Williams R. Urothelium derived inhibitory factor(s) influences on detrusor muscle contractility in vitro. *Br J Pharmacol* 2000; **129**: 416–9
- 26 Finney SM, Stewart LH, Gillespie JI. Cholinergic activation of phasic activity in the isolated bladder: possible evidence for M3- and M2- dependent components of a motor/ sensory system. *BJU Int* 2007; **100**: 668–78
- 27 Gillespie JIM, Markerink-van Ittersum M, de Vente J. Sensory collaterals, intra-mural ganglia and motor nerves in the guinea pig bladder: evidence for intramural neural circuits *Cell Tissue Res* 2008 (in press)
- 28 Streng T, Hedlund P, Talo A, Andersson KE, Gillespie JI. Phasic non-micturition contractions in the bladder of the anaesthetised and awake rat. *BJU Int* 2006; **97**: 1094–101

Correspondence: Steven Finney, Department of Urology, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, UK.
e-mail: s.mfinney@doctors.org.uk

Abbreviations: CGRP, calcitonin gene-related peptide; IF_{init} , frequency of transient contractions immediately after a volume increase; F_{ss} , frequency of transient contractions at steady state; P_{shift} , shift in underlying pressure; ChAT, choline acetyltransferase; NF, neurofilament protein.